Universidad Autónoma de Madrid

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INDUCIBLE AND ACQUIRED ANTIBIOTIC RESISTANCE IN *STENOTROPHOMONAS MALTOPHILIA*

Memory presented by

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ABSTRACT

Abstract

Antibiotic resistance is a global public health problem, which is aggravated by the emergence and spread of multidrug resistant (MDR) bacteria, especially Gram-negative bacterial species. Bacteria can present intrinsically low susceptibility to antibiotics as a result of the presence of resistance determinants encoded in their genomes. Besides, they can also become resistant through the acquisition of resistance genes through horizontal gene transfer, as well as by the selection of genetic mutations. Transient resistance can also be achieved as a consequence of a temporal induction of the expression of some of these resistance mechanisms, such as MDR efflux pumps.

Stenotrophomonas maltophilia is a Gram-negative opportunistic pathogen with an environmental origin and associated with infections in compromised patients, including those with cystic fibrosis and with other underlying pathologies. This bacterium exhibits an intrinsic low susceptibility to multiple antimicrobial compounds. Particularly, MDR efflux pumps from the resistance nodulation division (RND) family are among the most relevant determinants contributing to the intrinsic and acquired antibiotic resistance of *S. maltophilia*.

The role of RND efflux pumps in transient resistance has not been studied in *S. maltophilia* in detail. Hence, in this thesis, we aim to analyse the contribution of two *S. maltophilia* RND efflux pumps in transient resistance to antibiotics. To that goal, two screenings using fluorescence-based strains and a broad variety of compounds have been performed in order to find inducers of the expression of SmeVWX and SmeYZ efflux pumps. Inducer compounds were identified for both efflux systems, pointing that *smeVWX* expression is likely induced by the thiol-reactivity of the compounds, while *smeYZ* is induced by molecules that inhibit protein synthesis. The role of these identified inducers in transient resistance to antibiotics was also confirmed.

With the aim of defining new mechanisms involved in the acquisition of mutation-driven resistance to antibiotics, as well as to decipher the evolutionary trajectories towards such resistance, *S. maltophilia* was submitted to experimental evolution in the presence of increasing concentrations of the antibiotics ceftazidime or tigecycline. Whole-genome sequencing of the final-step populations revealed that SmeH, the transporter protein of the SmeGH efflux pump, is an important contributor towards ceftazidime resistance acquisition. Amino acid substitutions

in this efflux protein do not give rise to a fitness burden. However, they modify the susceptibility against other antimicrobials, possibly by producing changes in the access and binding of the substrate. Conversely, the first step towards the acquisition of tigecycline resistance is the overexpression of the SmeDEF efflux pump through mutations in the *smeT* gene, which encodes the transcriptional repressor of this efflux system. Besides, mutations in genes related to the ribosome (the tigecycline target), and to the lipopolysaccharide biosynthesis and membrane homeostasis, were also found mutated along the evolution period in the different evolved lineages. These mutations, which lead to cross-resistance to several antibiotics and collateral susceptibility to fosfomycin, impose a fitness cost for the *S. maltophilia* populations.

Overall, the presented results highlight the relevance of the *S. maltophilia* RND efflux pumps, since they play a fundamental role not only in intrinsic resistance, but also in the acquired resistance through mutations and in transient resistance through their overexpression under specific stress conditions.

Resumen

La resistencia a los antibióticos es un problema de salud global que se ha visto acentuado debido a la emergencia y diseminación de bacterias multirresistentes (MDR), en especial, bacterias Gram-negativas. Las bacterias pueden presentar, intrínsicamente, una baja sensibilidad a los antibióticos debido a la presencia de mecanismos de resistencia codificados en sus genomas. Además, la adquisición de genes de resistencia a través de eventos de transferencia horizontal de genes, así como de mutaciones genéticas, también pueden hacer que las bacterias se hagan resistentes. La resistencia transitoria a los antibióticos también puede ocurrir como consecuencia de una inducción temporal de la expresión de algunos de estos mecanismos de resistencia, como pueden ser las bombas MDR de expulsión múltiple de drogas.

Stenotrophomonas maltophilia es un patógeno oportunista Gram-negativo de origen ambiental y asociado a infecciones en pacientes comprometidos, incluyendo aquellos que padecen fibrosis quística u otras patologías subyacentes. Esta bacteria es intrínsecamente poco sensible a múltiples compuestos antimicrobianos. Particularmente, las bombas de expulsión múltiple de drogas pertenecientes a la familia *resistance nodulation division* (RND) se encuentran entre los elementos más importantes que contribuyen a la resistencia intrínseca y adquirida de *S. maltophilia*.

El papel de las bombas de expulsión de drogas RND en la resistencia transitoria no ha sido estudiado en detalle en *S. maltophilia*. Así pues, en esta tesis nos planteamos analizar la contribución de dos de las bombas RND de esta bacteria en la resistencia transitoria a los antibióticos. Para ello, se han llevado a cabo dos escrutinios utilizando cepas biosensoras y una amplia variedad de compuestos con el objetivo de encontrar inductores de la expresión de las bombas SmeVWX y SmeYZ. Se identificaron compuestos inductores para ambos sistemas de bombeo, cuyo modo de acción indica que la expresión de *smeVWX* es inducida, probablemente, por la capacidad de los compuestos de reaccionar con grupos tiol, mientras que la expresión de *smeYZ* es inducida por moléculas capaces de inhibir la síntesis de proteínas. El papel de los compuestos inductores identificados en la resistencia transitoria a los antibióticos también fue confirmado.

Con el fin de identificar nuevos mecanismos implicados en la adquisición de resistencia a los antibióticos mediante mutaciones, así como de determinar las trayectorias evolutivas hacia dicha resistencia, se han realizado evoluciones experimentales en presencia de concentraciones crecientes de los antibióticos ceftazidima o tigeciclina. La secuenciación masiva de los genomas pertenecientes a la última etapa de la evolución, reveló que SmeH, la proteína transportadora de la bomba SmeGH, es un importante contribuyente a la adquisición de resistencia a ceftazidima. Los cambios de aminoácido seleccionados en esta proteína no conllevan un coste de *fitness*. Sin embargo, modifican la sensibilidad bacteriana a otros compuestos antimicrobianos, posiblemente mediante cambios en el acceso y la unión del substrato. Por otro lado, el primer paso hacia la adquisición de resistencia a tigeciclina es la sobre-expresión de la bomba de expulsión de drogas SmeDEF debido a mutaciones en el gen *smeT*, el cual codifica para el represor transcripcional de este sistema de bombeo. Además, algunos genes relacionados con el ribosoma (diana de la tigeciclina), así como con la síntesis del lipopolisacárido y con la homeostasis de la membrana, también se encontraron mutados a lo largo del periodo de evolución de las diferentes poblaciones. Dichas mutaciones, las cuales conducen a la resistencia cruzada a varios antibióticos y a la sensibilidad colateral a fosfomicina, suponen un coste de *fitness* para las poblaciones evolucionadas de *S. maltophilia*.

En conjunto, los datos presentados resaltan la importancia de las bombas de expulsión múltiple de drogas de la familia RND de *S. maltophilia*, debido a su contribución no sólo a la resistencia intrínseca, sino también a la resistencia adquirida mediante mutaciones, así como en la resistencia transitoria a través de su sobre-expresión ante condiciones de estrés específicas.

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INTRODUCTION

1. Introduction

1.1. Antibiotic resistance: a global threat

Antibiotics are among the most successful compounds with a therapeutic application developed in the history of medicine. Since their discovery and introduction in the last century, they have saved countless lives by contributing not only to the treatment of life-threating infectious diseases, like pneumonia, meningitis or sepsis caused by wound infections, but also to prevent infections that might occur, for instance, after a surgical procedure [1]. The "golden era" of antibiotics ranged from the 1950s to 1970s and gave rise to the discovery of many novel antibiotic classes [2]. Unfortunately, shortly after the introduction of most antibiotics, resistant strains that were able to overcome the toxic effects of these compounds appeared (Figure 1).

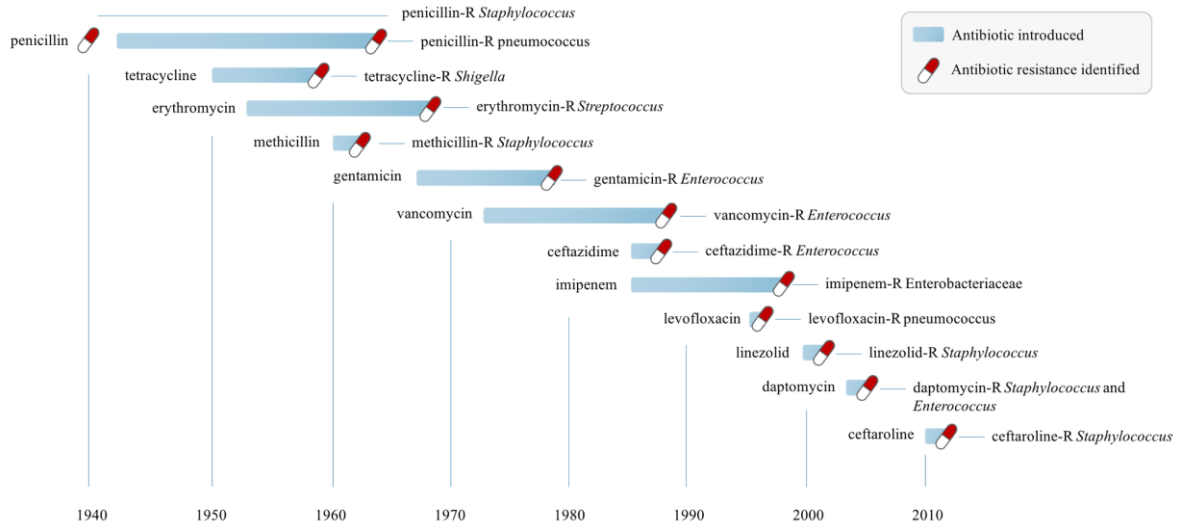

Figure 1. Timeline showing the introduction of several antibiotics and identification of resistance. For some of them, as methicillin, levofloxacin, linezolid, daptomycin or ceftaroline, resistance emerged little after the introduction of the antibiotic. Source: Centers for Disease Control and Prevention.

Antibiotic resistance occurs when a drug loses its ability to inhibit the growth of a microorganism in an effective way [3]. In the last decades, we have witnessed a significant increase in the emergence of antibiotic resistant bacterial strains. Although this resistance results mainly as a consequence of a selection pressure set on susceptible bacteria by the use of antimicrobial drugs, both for treating human infections and in animal production, several social and administrative factors, as noncompliance, self-medication, or the loss of interest in the discovery and introduction of new antimicrobial drugs by the pharmaceutical companies, have

also increased the problems associated with the emergence and spread of antibiotic resistance [4,5]. The issue is more relevant because some bacterial pathogens have become resistant to many antimicrobial agents, showing a multidrug-resistant (MDR) phenotype [6,7]. MDR bacteria have become one of the most important current threats to public health, being associated with high mortality rates. Considering Europe, it is estimated that MDR pathogens are responsible of 33,000 deaths per year [8]. It is important to highlight the fact that MDR bacteria can be found across the animal, human and environmental settings, existing a continuous interchange of these pathogens among the three niches and a consequent spread of their resistance genes [9,10]. The fact that some of these MDR bacteria are able to adapt to several hosts and survive in a broad range of potential niches is of utmost concern, and could increase their ability to acquire new resistance or virulence determinants while fitness is maintained [10].

1.2. Definitions of antibiotic resistance

Classically, antibiotic resistance has been defined from a practical point of view that categorize bacteria as susceptible or resistant on the basis of the feasibility of treating the infections they produce. Thus, from a clinical viewpoint, definition of resistance is based on the minimum inhibitory concentration (MIC) breakpoints, which are clinically relevant values when deciding which antibiotic to use in therapy [11]. Nevertheless, this definition is not adequate for non-human-associated bacteria or other antimicrobial compounds that are not used in clinics, for which no established breakpoints are available. Besides, the clinical definition of resistance does not take into account low-level resistance, which can be also of importance during the first steps of high-level drug resistance acquisition [12]. Therefore, in order to study different processes, as resistance evolution or functional genomics of antibiotic resistance, other more suitable definitions are employed.

The epidemiological definition of antibiotic resistance is based on the epidemiological cut-off values for resistance (ECOFFs), which have been proposed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) to describe the upper MIC limit of the wild-type population of a particular species [13,14]. Those isolates whose MICs are above this breakpoint, are considered resistant even if the MICs do not reach the clinical breakpoint. This definition can be used to determine low-level resistance, as well as the breakpoints for other compounds different than antibiotics, as biocides, for which a clinical definition of resistance is not available [15]. However, since this epidemiological definition is based on the analysis of a

large number of independent isolates, it is not appropriate for the study of single genes or mutations in a particular isolate.

In these cases, a gene-centric definition of resistance (operational definition) is used [14]. This third definition, which is the one that will be employed in this thesis when defining resistant or susceptible strains, is based on the comparison of a wild-type strain with a mutant strain, or with a strain that harbours a resistance gene acquired through horizontal gene transfer (HGT). If this strain has a MIC value higher than the one obtained for the parental strain for the same antimicrobial, it is considered resistant. Although this definition does not include predefined breakpoints, it is the most appropriate for the analysis of the role of a specific gene in the susceptibility to a given antibiotic [14,16].

1.3. Types of antibiotic resistance and molecular mechanisms

The inhibitory activity of a given antibiotic relies mainly on two basic parameters: the amount of antibiotic that reaches the target inside the cell, and the efficiency of the antibiotic/target interactions. Thus, antibiotic resistance can be achieved either by reducing the intracellular concentration of the drug, for instance, through the activity of MDR efflux pumps, antibiotic-modifying enzymes or alteration of the pathway for drug entrance; or by reducing the affinity of the interaction through target site alteration by mutations, among others [17].

All of these mechanisms are involved in the intrinsic, acquired or adaptive resistance of bacterial pathogens (described hereunder), particularly in Gram-negative bacterial species that are resistant to several antibiotics and whose raise has become a serious challenge in all parts of the world [18,19]. Some of these determinants can contribute to the three types of resistance, and it is not the presence of just one of them which renders bacteria less susceptible to antibiotics, but the interplay among several of these resistance mechanisms.

1.3.1. Intrinsic resistance

The recent use of antibiotics for human therapy and farming is not the unique driving force towards antibiotic resistance of human pathogens. Some bacterial species with an environmental origin (such as *Pseudomonas aeruginosa* or *Stenotrophomonas maltophilia*),

where the antibiotic load is not as high, as occurs in hospitals, present an intrinsic low susceptibility against several antibiotics [20].

As discussed in the previous paragraph, bacteria are considered intrinsically resistant if the infections that they cause cannot be treated with a given antibiotic, based on the clinical definition [21]. However, all the elements that directly or indirectly contribute to antibiotic susceptibility, regarding the epidemiological and the operational definitions as well, and whose presence is independent of prior antibiotic exposure or HGT, define the "intrinsic resistome" [16]. All the chromosomally encoded genes that have been recently acquired as a consequence of the human use of antibiotics, are not encompassed in this definition [21]. Although the intrinsic resistome of a given species comprises a wide variety of genes [16,22-25], the most relevant causes of intrinsic resistance in Gram-negative bacteria, from a clinical point of view, are low uptake of antibiotic, lack of the target or presence of an intrinsically resistant allele, antibiotic inactivation, and efflux of the antibiotic (Figure 2) [17].

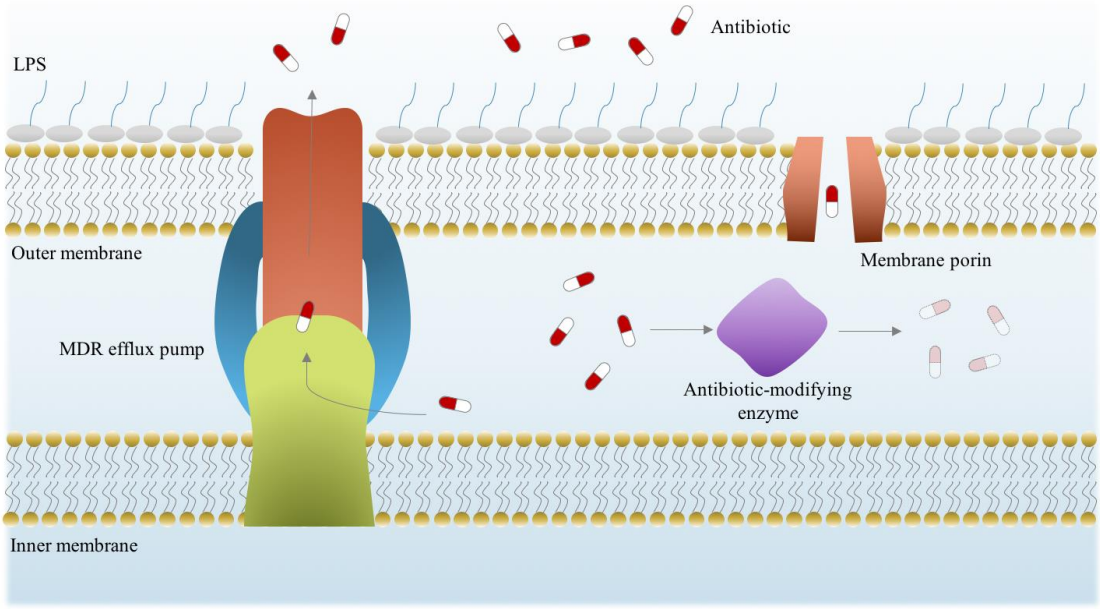

Figure 2. Schematic representation of the main intrinsic resistance mechanisms of Gram-negative bacteria. The presence of the lipopolysaccharide (LPS) in the bacterial outer membrane creates a barrier that prevents the passive diffusion of hydrophobic antibiotics. Those that manage to reach the periplasmic space, for instance, through membrane porins, can be counteracted by other mechanisms, such as inactivation by antibiotic modifying enzymes, or extrusion by multidrug resistance (MDR) efflux pumps.

The membrane of Gram-negative bacteria consists in an asymmetric bilayer of lipopolysaccharides (LPS) and phospholipids where specialized protein channels are found

embedded [26]. This LPS-structure confers the bacterial membrane a higher rigidity compared to a regular bilayer, which slows the passive diffusion of hydrophobic compounds, such as aminoglycosides, macrolides, rifamycins, novobiocin, fusidic acid and cationic peptides [26,27]. Besides low uptake of the drug, the absence of the target of a specific antibiotic is also a common example of intrinsic antibiotic resistance. For instance, the antistaphylococcal agent daptomycin is not effective against Gram-negative bacteria since the content of negatively charged phospholipids, which allows the insertion of daptomycin into the cytoplasmic membrane, is substantially lower compared to Gram-positive bacteria [28]. In other occasions, bacteria have the specific target for an antibiotic, but it is an intrinsically resistant allele, which makes this bacterial species resistant to such antibiotic. A good example for illustrating this situation is the MurA enzyme, involved in the bacterial cell wall peptidoglycan synthesis and the target of the antimicrobial agent fosfomycin [29]. Bacterial species carrying a regular MurA enzyme with a cysteine in its active site, such as *Escherichia coli*, are susceptible to this antibiotic; however, other bacterial pathogens, such as *Chlamydia* and *Borrelia burgdorferi*, harbour MurA variants without a cysteine in the enzyme active site, without previous selection under fosfomycin exposure, that make these bacteria intrinsically resistant against this drug [30,31].

Another effective way to resist the toxic effect of an antibiotic is through its inactivation. This can be achieved by both antibiotic hydrolysis performed by specific enzymes, such as beta-lactamases that hydrolytically cleave the beta-lactam ring of beta-lactam antibiotics [32], or by the modification of the drug through the addition of chemical groups, which results in structural alterations that impair target binding. This last category encompasses the group of transferases, including aminoglycoside acetyltransferases, macrolide kinases or nucleotidyltransferases [33]. Although many of these antibiotic-modifying enzymes are associated with resistance plasmids, they can also be encoded by chromosomal genes, thus contributing to the bacterial intrinsic resistance. One example is the *Acinetobacter baumannii* non-inducible AmpC-type cephalosporinase that confers, at basal levels of expression, resistance against several beta-lactam drugs [34].

Decreasing the intracellular concentration of an antibiotic is another common strategy that bacteria follow in order to prevent the toxic compound to efficiently reach its target, which is usually located inside the cell. In this respect, bacterial MDR efflux pumps are major contributors to the intrinsic resistance of Gram-negative bacteria since they actively extrude a

wide variety of antibiotics, as well as other toxic compounds, outside the cell [35]. The resistance nodulation division (RND) family is the best characterized in Gram-negatives. The inner membrane protein (IMP) of these three-component complexes works in conjunction with an outer membrane protein (OMP), and a periplasmic membrane fusion protein (MFP) that links the IMP and the OMP [36]. Some examples are MexAB-OprM from *P. aeruginosa* [37], AcrAB-TolC from *E. coli* [38], or SmeYZ from *S. maltophilia* [39], whose basal levels of expression are sufficient for contributing to the intrinsic antimicrobial resistance of these microorganisms.

1.3.2. Acquired resistance

Acquisition of resistance to antibiotics can be mediated by chromosomal DNA mutations or by transfer and acquisition of new resistance genes from bacteria of the same or different genera or species (Figure 3) [40]. Regarding genetic mutations, these can lead to modifications of the antibiotic target, so that the affinity between antibiotic and its target is diminished and the binding is not carried out. A common example of antibiotic target modification is the one occurring in the bacteria penicillin binding proteins (PBPs), leading to beta-lactams resistance. For instance, mutations in the *Neisseria gonorrhoeae* PBP 1-coding gene *ponA*, have been found in high-level penicillin-resistant clinical isolates [41].

In addition to target modification, chromosomal mutations can alter the expression of several resistance determinants, as those happening in genes encoding the regulators of elements associated to antibiotic resistance, as MDR efflux pumps, antibiotic inactivating enzymes or porins [42]. MDR efflux pumps, apart from contributing to the bacterial intrinsic resistance, can also acquire mutations that result in an ameliorated extrusion of the antibiotics, led by either an increase of their expression level or, less commonly, by structural changes that make the efflux more efficient. Overexpression of efflux pumps is achieved mainly through mutations in the genes encoding their transcriptional local regulators. Well-known examples are the *P. aeruginosa* Mex systems from the RND family, such as MexAB-OprM, whose overexpression, and the consequent resistance phenotype, is due to mutations in the genes encoding its repressors, specifically *mexR*, *nalC* or *nalD* [43-45]. Similarly, acquisition of mutations in the *S. maltophilia* efflux pumps regulators *smeT* and *smeRv*, leads to the overproduction of the SmeDEF and SmeVWX systems, respectively, causing resistance to several antimicrobial agents [46,47]. Although the vast majority of mutations affecting efflux pumps occur in regulatory elements, in some other cases, mutations arise in the transporter

protein of the efflux pump, making the antibiotic extrusion more efficient. Examples of these types of mutations have been described in AcrB from *E. coli* [48] or in MexY from *P. aeruginosa* [49].

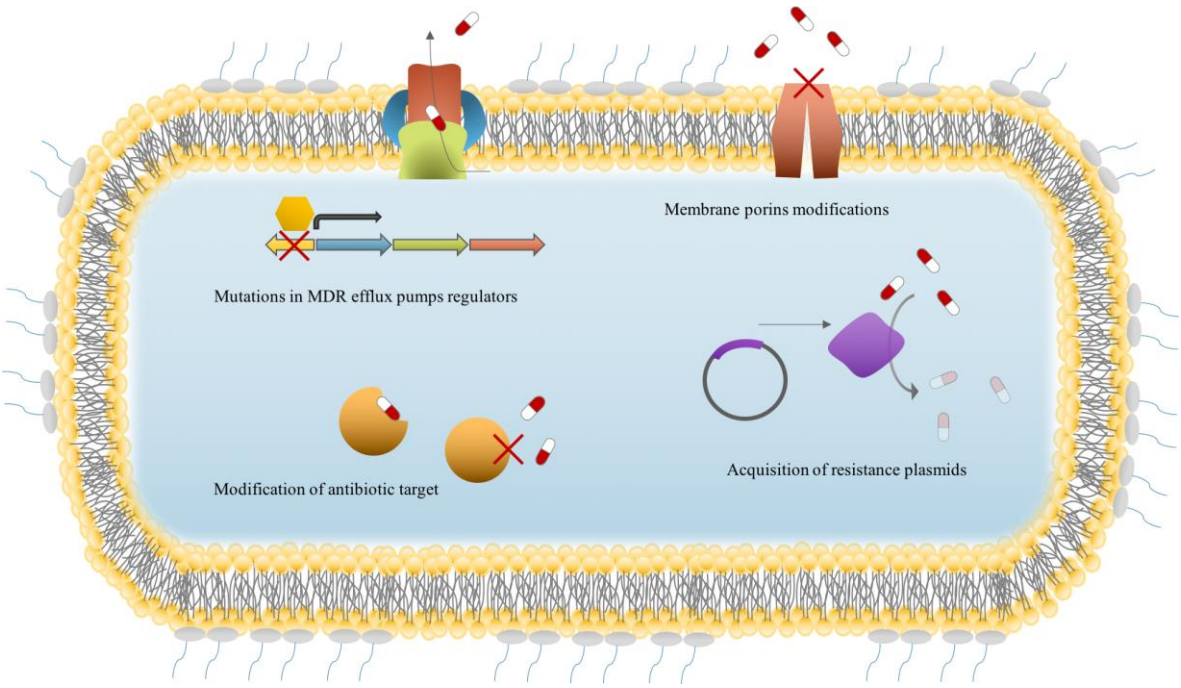

Figure 3. Schematic representation of the best studied acquired resistance mechanisms to antibiotics. Bacteria can acquire resistance through genetic mutations in the local transcriptional regulators of MDR efflux pumps, leading to the overexpression of these systems and antibiotic extrusion. Membrane porin-coding genes may also acquire mutations causing porin modification and prevention of antibiotic entrance. Mutations affecting the antibiotic target can also drive to antibiotic resistance caused by antibiotic-target binding inhibition. Acquisition of plasmids encoding resistance genes, such as antibiotic-modifying enzymes, is also an important mechanism of acquired resistance.

Resistance mutations can also be acquired in the membrane porin-coding genes, decreasing or preventing the antibiotic entrance inside the cell. These mutations can affect the size of the porin, its expression level, or even can result in porin loss. For instance, *E. coli* isolates from a patient subjected to a treatment with various antibiotics present changes in the OmpC porin that result in the modulation of resistance to the beta-lactam cefotaxime [50]. Besides these canonical mechanisms, several studies have demonstrated that resistance can be acquired through mutations in many more genetic elements, including transcriptional regulators, enzymes that participate in different bacterial processes, or motility-related genes, which can be involved, directly or indirectly, in the susceptibility to an antimicrobial compound [51-54].

Bacteria can also become resistant through the acquisition of different antibiotic resistance genes, either through transformation or via the interchange of genetic elements, such as plasmids [55,56], bacteriophages [57] and/or transposons [58] that carry antibiotic resistance determinants. Plasmids carrying extended-spectrum beta-lactamases (ESBLs) in different *Enterobacteriaceae* species [59], or the genomic islands present in *Salmonella enterica* that carry a class 1 integron containing transferable resistance genes [60], are examples of horizontal acquisition of resistance. Although the acquiring of external resistance genes is a very relevant mechanism concerning antibiotic resistance spread across different bacteria and ecosystems, it will not be discussed in this thesis.

1.3.3. Transient resistance

The third category of antibiotic resistance is transient resistance, also known as phenotypic or adaptive resistance. It can be defined as a temporary alteration in the bacterial physiology as a response to environmental signals/cues, conferring bacteria an increased capacity to overcome an antimicrobial challenge [42]. In contrast with intrinsic and acquired resistance, which are stable and can be transmitted vertically or horizontally, respectively, to consecutive generations, transient resistance is not inheritable and generally reverts upon removal of the triggering signal or condition (Figure 4) [61]. Transient resistance is particularly relevant since there are many signals that can act as inducers, including antimicrobials, oxidative or nitrosative stress, temperature, mode of growth (biofilm vs. planktonic), or even host-related compounds, compromising the course and treatment of bacterial infections [61-64].

Several molecular mechanisms are known to be behind transient resistance. For instance, magnesium limitation can be sensed by the two-component system (TCS) PhoPQ in *S. enterica*, activating another TCS, PmrAB, which leads to the upregulation of a LPS-modification operon (*pmr*). This transient upregulation reduces the net negative charge of the cell surface, thus limiting the interaction with positively charged antimicrobials and polymyxin B [65]. MDR efflux pumps are also important transient resistance determinants since their expression can be triggered by a diverse range of molecules and conditions. Some examples are the RND efflux pumps AcrAB from *E. coli*, and CmeABC from *Campylobacter jejuni*, whose expression is triggered by bile salts, conferring resistance to diverse antibiotics [66,67]. Similarly, expression of the *S. maltophilia* SmeDEF efflux system is induced by plant-derived flavonoids [68], as well as by the biocide triclosan, reducing the bacterial

susceptibility to quinolones [69]. Inducible beta-lactamases are also a major mechanism involved in transient resistance in many Gram-negative bacteria. The expression of these enzymes, such as AmpC, can be induced indirectly by the action of particular beta-lactam antibiotics, leading to an unsuccessful therapeutic outcome [70]. Other mechanisms, such as outer membrane modification through the modulation of porins expression, have also been reported to be involved in transient antibiotic resistance [71].

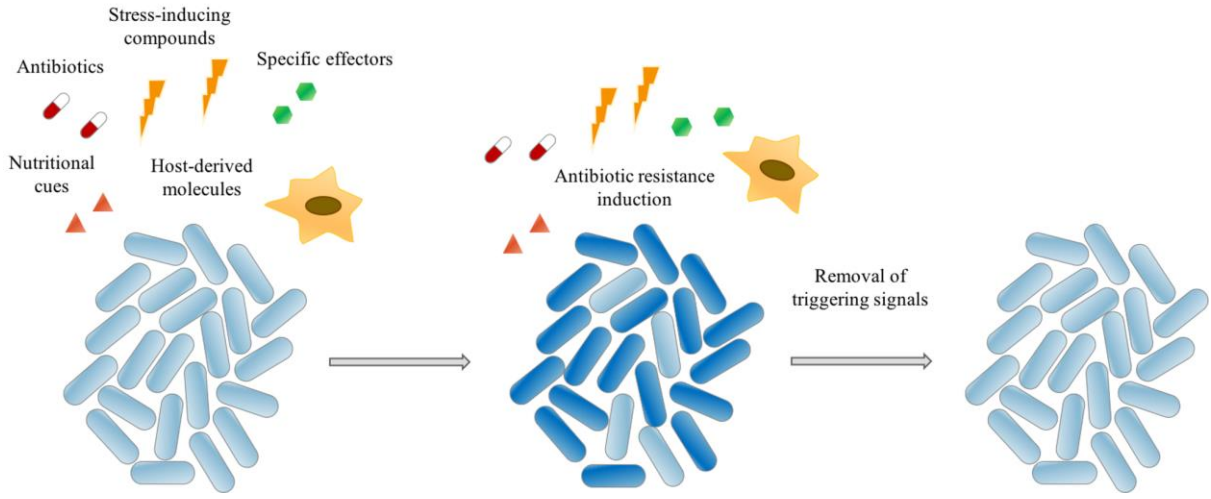

Figure 4. Induction of transient resistance to antibiotics. Changes in several environmental conditions, such as the presence of antimicrobial molecules, induction of the stress response or growth conditions, can lead to the induction of transient resistance to antibiotics (dark blue rods). However, this phenotype is reversible when the triggering signal is removed, thus recovering the susceptible phenotype (light blue rods).

Because of its temporary nature, transient resistance is difficult to detect and its investigations is limited to a few microorganisms and inducers/conditions. Nevertheless, since bacteria can encounter some of the above-mentioned inducer molecules or conditions during the course of an infection, which could lead to a transitory resistance situation, transient resistance study should not be disregarded.

1.4. RND efflux pumps: functions and regulation

Among all the resistance determinants, MDR efflux pumps are particularly relevant. There exist six major families of bacterial MDR efflux pumps: the ATP-binding cassette (ABC), the major facilitator superfamily (MFS), the multidrug and toxin extrusion (MATE), the small multidrug resistance (SMR), the proteobacterial antimicrobial compound efflux (PACE), and the previously mentioned resistance nodulation division (RND) family [72]. The

latter family is specific to Gram-negative microorganisms and forms part of a tripartite complex which spans across the two membranes of these Gram-negative bacteria [73]. RND efflux pumps are powered by the electrochemical potential of protons, or proton motive force, in order to transport the substrates across the cell membrane [74]. It is proposed that RND membrane transporters, that is, the periplasmic proteins, can form homotrimers, and each monomer has varying conformations during substrate transport, named *access*, *binding*, and *extrusion*, as described for the *E. coli* AcrB transporter. Thus, the different compounds are exported following an orderly conformation change in the three monomers through a rotation mechanism that is driven by the protons translocation (Figure 5) [75].

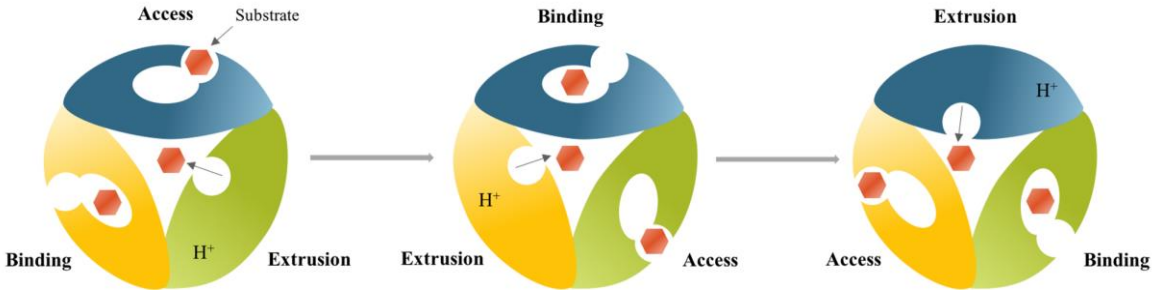

Figure 5. Schematic representation of the functional-rotation mechanism of substrate export mediated by the homotrimers of the RND transporter proteins. Each monomer (blue, green and yellow) changes its conformation going through three states: *access*, in which the substrate enters into the access pocket or vestibule of the transporter; *binding*, in which the substrate accommodates inside the binding pocket; and *extrusion*, in which the substrate exits through a funnel towards the outer membrane protein for being extruded outside of the cell. These conformational changes are coupled to the proton translocation (Adapted from Murakami, 2008 [76]).

Although the majority of the studies regarding RND efflux pumps are focused on their role as antibiotic resistance determinants (they can contribute to the three types of resistance), these efflux systems are able to extrude other structurally unrelated compounds, including heavy metals, organic pollutants, or bacterial metabolites, among others. This fact highlights the role of efflux pumps in the detoxification of damaging endogenous and exogenous compounds [77]. RND-type efflux pumps are also required for colonization and propagation during host infection, helping as well to extrude innate host defences. For instance, *P. aeruginosa* lacking MexAB-OprM efflux pump is unable to invade epithelial cells, a function that is restored after efflux pump complementation [78]; and some *Vibrio cholera* RND efflux pumps are required for virulence factors' production and intestinal colonization [79]. Other important roles that have been identified are their involvement in the trafficking of quorum-sensing signals [80], and in plant-bacteria interactions [81].

Since RND efflux pumps are involved in many physiological processes and demonstrate a wide substrate range, a regulatory control over the expression of these systems is to be expected. Modulation of the expression of RND efflux pumps is often mediated by local or global regulators, including TCSs, transcription and post-transcription factors (repressors and activators) [82], and even small proteins that can act as regulatory elements [83,84]. Besides, certain environmental conditions, as stress situations, or the presence of particular signals/compounds, can also transiently regulate the expression of RND efflux pumps, as mentioned in the previous paragraph. Therefore, understanding RND efflux pump regulation is a noteworthy target to be addressed, since alterations in the regulatory systems, such as mutations in a transcriptional repressor, or the presence of inducer molecules, can lead to their overexpression and a consequent extrusion of antibiotics.

1.5. Effects of the acquisition of antibiotic resistance on bacterial physiology

1.5.1. Fitness cost

It is generally accepted that genetic mutations and/or acquisition of antibiotic resistance determinants are associated with metabolic changes, including fitness cost. This vision is based on the fact that mutations that drive to resistance might occur in genes that are important for bacterial physiology [85,86]. For instance, mutations can arise in genes that regulate the expression of antibiotic detoxification elements, such as MDR efflux pumps, as well as in the antibiotic targets or transporters. Since these elements are frequently well conserved and are important determinants for bacterial physiology, their mutation can lead to a poor functioning and, as a consequence, to a physiological burden that implies a disadvantage in comparison with the wild-type strain [87]. Moreover, mutations leading to the overexpression of some antibiotic resistance determinants, as efflux pumps or antibiotic-inactivating enzymes, are physiologically costly, since, in a drug-free environment, a constant overproduction of these systems will drive to a non-needed metabolic burden [88]. Furthermore, acquisition of resistance genes, for instance, through HGT, could also imply a reduced bacterial fitness as a result of replication, transcription and translation of the newly-acquired genes [89]. This fitness cost, usually observed as a reduction of growth rate, is a key biological parameter that determines the evolution of resistance.

1.5.2. Compensation mechanisms for fitness costs alleviation

Since antibiotics act as a selective pressure towards resistance acquisition, the occurrence of a fitness cost associated with this resistance can lead to the idea that a reduction in the antibiotics usage would suppose an advantage for the fitter susceptible ancestor, which would replace the resistant strain in a drug-free environment [85,90]. However, there exist some exceptions where resistance happens to be neutral or even beneficial [91,92]. For instance, it has been reported that acquisition of resistance in *P. aeruginosa*, *A. baumannii* and *V. cholerae* is associated with an increased fitness *in vivo* in different infection settings [93].

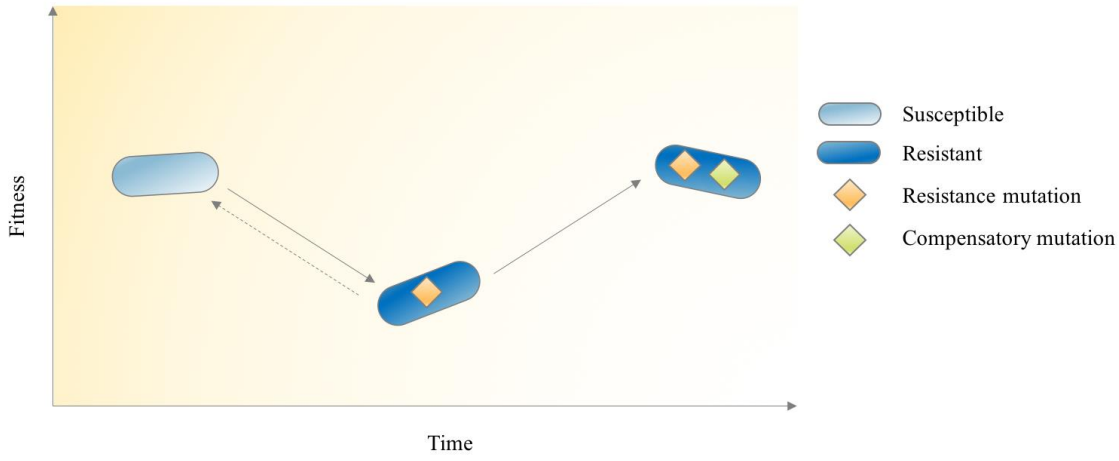

Figure 6. Compensation of fitness costs derived from antibiotic resistance. Acquisition of antibiotic resistance mutations may impose a fitness impairment in the absence of the selective pressure (antibiotic). These costs can be compensated, withouth compromising resistance, through the acquisition of compensatory mutations. An unlikely situation would be the reversion towards the wild-type susceptible genotype (dotted line).

Although these no-cost mutations can contribute to the spread of some resistant mutants, other alternatives allow the resistant bacteria to remain even in the absence of selection. This can occur through a genetic reversion to the parental strain, although this is a rare event [85,94], or through the acquisition of second-site mutations that compensate the fitness costs imposed by a resistance mutation (Figure 6) [95,96]. Compensation can occur through mutations that restore the fitness through the substitution of the affected function with an alternative activity, or by restoring, directly or indirectly, the efficiency of the concerned function [97,98]. Thus, compensatory mutations can arise within the same gene that caused the resistance phenotype by recovering the protein functionality, or can occur in other genes. Compensatory evolution has been observed in both *in vitro* [95], *in vivo* [97,99], and in clinical studies [100], showing that

compensatory mutations are different according to each type of experimental model, thus demonstrating that fitness costs are highly dependent on the bacterial habitat [99].

In occasions, compensation can also be caused by another resistance mutation. For instance, a mutation in *rpsL*, causing streptomycin resistance, can be compensated by different mutations that result in rifampicin resistance in *E. coli*. Besides conferring resistance to a new antibiotic, which would complicate the antibiotic treatment, the fitness of the double mutants is greater than the fitness of at least one of the single mutants [101].

Bacteria can also compensate the fitness costs associated with antibiotic resistance without the need of mutations. Examples of this situation are MDR efflux pump-overexpressing mutants of *P. aeruginosa* that are able to rewire their metabolism in order to avoid the fitness costs derived from the MDR efflux pumps overexpression [102]; or overexpression of the rRNA methylase TlyA as a mechanism to increase fitness and reduce the deleterious effects of a resistance mutation in mycobacteria [103].

1.5.3. Cross-resistance and collateral sensitivity

Acquisition of antibiotic resistance, beyond involving eventually a fitness cost, is usually accompanied by other changes in response to certain environments. One phenomenon that might occur after acquiring resistance to one drug is the development of resistance, or the enhancement of sensitivity, to a different drug(s) simultaneously, which is called cross-resistance or collateral sensitivity, respectively [104]. These changes in the bacterial susceptibility to other antimicrobial compounds can be caused by various mechanisms. For instance, a mutation that alters the expression of an efflux pump, caused by the exposure to an specific antibiotic, would favour cross-resistance against other compounds that are substrates of such efflux pump, including clinically relevant antibiotics [105]. While cross-resistance is a problematic outcome regarding the treatment of bacterial infections, collateral sensitivity in the clinics could be exploited through combination therapy or by the use of temporal cycling of different antibiotics. Thus, an understanding of how the evolution of resistance towards an antibiotic can affect the susceptibility to other drugs is of relevance [106].

1.6. Antibiotic resistance evolution

As pointed out above, resistance can evolve rapidly and through several mechanisms, having important consequences for human health. Thus, predicting and understanding the factors that drive and constrain the evolution of resistance is essential for minimizing the probability that antibiotic resistance arises [107].

1.6.1. Factors that determine the evolution of antibiotic resistance

Bacteria possess an abundance of mechanisms that can potentially confer antibiotic resistance. However, the number of the resistance mechanisms that are finally selected is limited. This limitation, which is determined by the ecological dimension of resistance, defines the evolutionary trajectories towards antibiotic resistance [108]. Evolutionary trajectories, as well as the spread and maintenance of antibiotic resistance in a bacterial population, are driven by the interplay of several factors (Figure 7) [109]: i) the rate at which resistance and mutations arise in a population (mutation supply rate), which is determined by the population size, rate of mutation and HGT events; ii) the level of resistance that is conferred by the resistance mechanism, which depends on the resistance determinant itself and the conditions under which resistance is analysed. In some situations, the level of resistance changes is dependent on the environmental conditions [110]. For instance, urine at neutral pH increases the MIC for fosfomycin in *E. coli* clinical isolates; however, acidic urine and anaerobiosis conditions render these isolates more susceptible to this antibiotic [111]; iii) the relative fitness of the resistant mutant at different drug concentrations. As previously discussed, several antibiotic resistance mechanisms impose a fitness cost to the resistant mutant in the absence of the selective pressure, although some resistance mutations might be neutral or even beneficial [92]. Thus, the relative fitness is a key parameter for assessing the evolutionary success of the resistant mutant in the host population or other environments [112]; iv) the strength of the selective pressure, which would also have an influence on the rate of emergence of mutations and the type of mutants. For example, the mutations that are selected at high level of antibiotic concentrations would differ from those selected at non-lethal drug concentration [113].

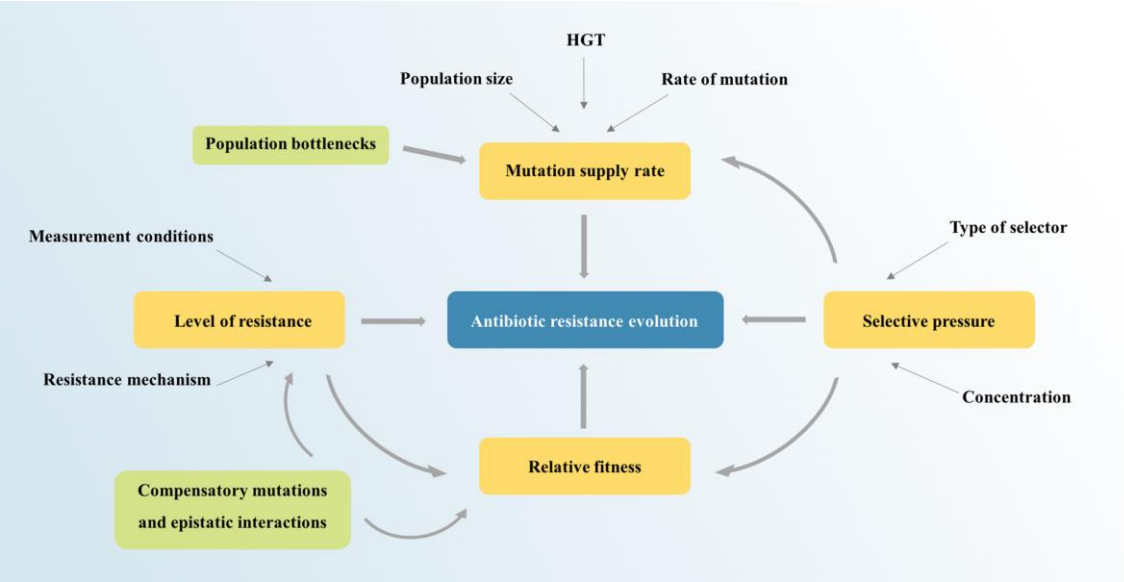

Figure 7. Interplay between the factors that influence the evolution of antibiotic resistance. Four basic factors (mutation supply rate, level of conferred resistance by the resistance mechanism, relative fitness of the resistant mutant, and strength of the selective pressure) have a direct effect on the evolution of antibiotic resistance. These factors are in turn influenced by other determinants. The emergence of compensatory mutations, the presence of epistatic interactions or population bottlenecks are also main determinants in the evolution of resistance to antibiotics.

In addition to these basic factors, other elements have also a strong impact on antibiotic resistance evolution. The genetic background has an influence on a mutation's effect, meaning that the effect of a mutation in a specific gene can fluctuate drastically depending on the presence or absence of another mutation in another gene [114]. This situation is referred to as "epistasis" or "epistatic interaction" and might have an effect on the level of resistance and on the relative fitness of the resistant mutant. Epistasis is considered positive, when the fitness of the double mutant is higher than the expected from the effects of the two single mutations; negative, when the fitness of the double mutant is lower than the expected from their effects when alone; or sign epistasis, in the case that the fitness of a mutation varies relying upon the genetic background [96]. The fact that later mutations in evolution are dependent on the earlier ones, illustrates a feature known as contingency. Thus, a mutation may be contingent on prior mutations and could constrain future mutations along the evolutionary trajectory, owing to epistasis [115,116].

Compensatory mutations are also key factors for antibiotic resistance evolution since they can restore the bacterial fitness without a loss of resistance [109]. The interactions between antibiotic resistance mutations and compensatory mutations are usually considered as sign epistasis, since these compensatory mutations are beneficial on the resistant strain but might be deleterious, or have no effect, on the parental strain background [117].

Finally, population bottlenecks are also major determinants affecting the evolution of resistance. Bottlenecks are events that dramatically reduce the original population size, for instance, the inoculum of a bacterial population for starting an infection. In some cases, population bottlenecks can eliminate certain genotypes from a gene pool, even if these genotypes are not associated with a low fitness [118]. Thus, these population-reducing events can constrain the evolutionary trajectories by restricting the mutation supply. The population size, the mutation rates and the fitness influence transmission bottlenecks [112].

1.6.2. Experimental evolution for predicting antibiotic resistance

If our purpose is to predict how resistance to an antibiotic will arise, it is necessary to know which resistance genes and mutations are most likely to confer resistance to the tested antibiotic. In addition, knowing which new genes might emerge and spread in the bacterial population, contributing to shorten the therapeutic lifetime of the antibiotic, is also required [107].

A frequent method for analysing the mechanisms involved in the antibiotic resistance evolution is performing *in vitro* evolution experiments in the presence of antibiotics. By this approach, bacterial populations are exposed to a known drug concentration sufficient to partially inhibit the growth of the parental strain, but low enough to allow spontaneous resistant mutants to survive [54]. In order to allow the selection of improved phenotypes, serial passages increasing the antibiotic concentration are performed for prolonged periods of time, that could range from days to years [54,119]. The rate at which the antibiotic concentration is increased, which reflects the rate of resistance evolution, can vary in time and with the use of different drugs [54].

With the aim of identifying the mutations involved in the acquisition of resistance, PCR amplification and sequencing of the suspected target genes can be performed; however, whole-genome sequencing (WGS) allows to detect new resistance mechanisms (Figure 8) [119]. Using this approach, it is possible to describe mutations that confer drug resistance before their

emergence in nature or clinics. Moreover, if these mutations appear in patients, their identification will allow to facilitate the diagnosis of resistant infections, as well as the measures for the most appropriate therapeutic treatment [120].

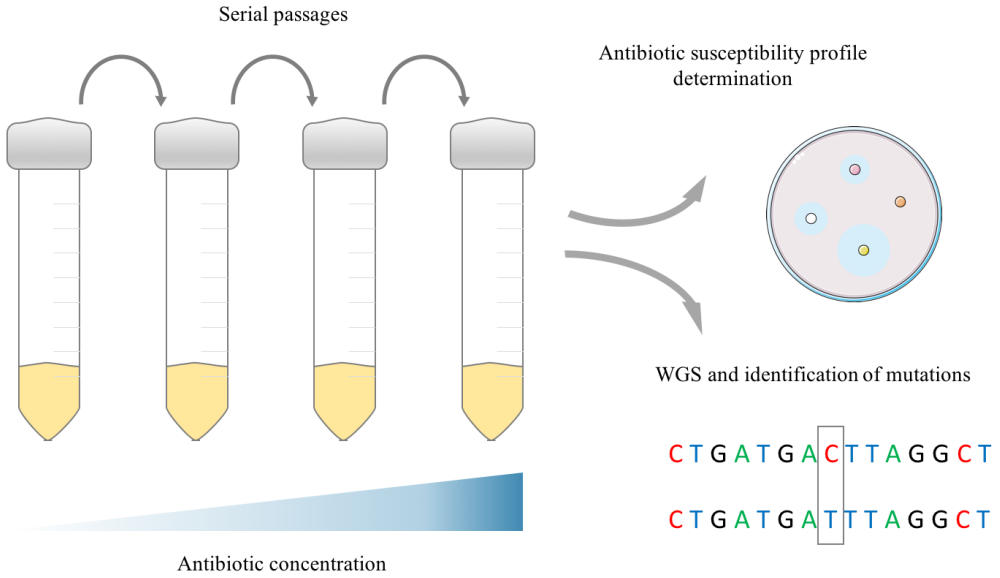

Figure 8. Schematics of an experimental evolution assay in the presence of antibiotic. A bacterial population is initially grown at the maximum antibiotic concentration that allows bacterial growth. Serial passages are performed by inoculating a bacterial culture dilution into fresh media for several generations while antibiotic concentration is increased in order to promote selection. At the end of the experiment, antibiotic susceptibility is determined and bacterial genomic DNA is extracted and whole-genome sequenced (WGS) with the aim of identifying resistance mutations.

Besides determination of resistance mutations, serial passages experiments are also useful for analysing and identifying compensatory mutations that ameliorate the fitness costs that might be imposed by other resistance mutations, as well as for the detection of epistatic interactions [119], which can strongly influence the evolution of resistance and thereby complicate its prediction [112]. Multistep experimental evolution also reveals the evolutionary trajectories towards resistance to a given antibiotic. The ability to predict the order of appearance of the mutations during the experimental evolution is also crucial to assess the relative importance of each genetic change towards resistance. Recurrent evolutionary patterns, such as the emergence of mutations in a preferred order, can provide some degree of predictability to an evolutionary process that, at first sight, might seem stochastic [54].

1.7. *Stenotrophomonas maltophilia*: an opportunistic pathogen

Among MDR Gram-negative bacteria, *S. maltophilia* has become a relevant nosocomial pathogen in the past years. Taxonomically, this bacterium belongs to a subclass of proteobacteria that was firstly described as *Pseudomonas maltophilia* at 1961 [121]. Further studies led to its reclassification as *Xanthomonas maltophilia* in 1983 [122], and finally, to *Stenotrophomonas maltophilia* in 1993 [123].

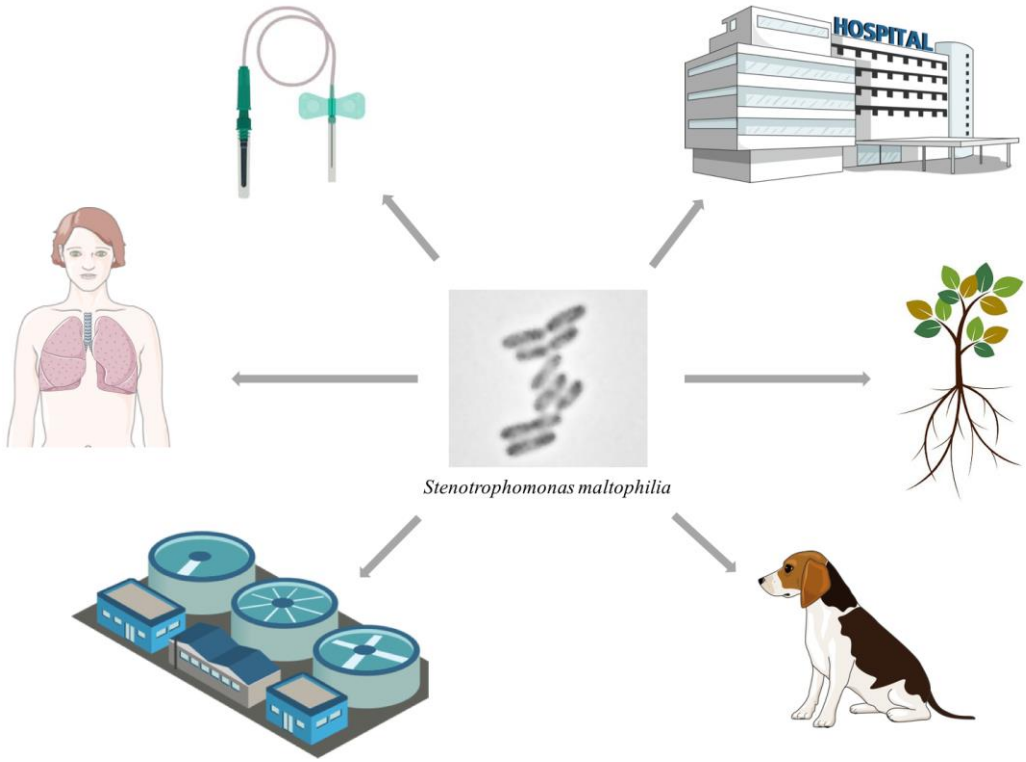

Figure 9. *S. maltophilia* as a ubiquitous nosocomial pathogen. *S. maltophilia* has been isolated from both environmental (root plants, animals, wastewater plants, soil) and clinical-associated sources (catheters, sink drains, disinfectants, endoscopes).

S. maltophilia is an environmental ubiquitous bacterium that has been isolated from several sources, both inside and outside the clinical setting (Figure 9). It has been recovered from habitats like plant roots [124], animals [125], wastewater plants [126], hand-washing soap [127] or food [128]. *S. maltophilia* is characterized by its ability to form bacterial biofilms, colonizing both abiotic (catheters, nebulizers or prosthetic devices), and biotic surfaces, including lung cells [129,130]. An interesting fact to have into account is that environmental and clinical *S. maltophilia* isolates do not form different phylogenetic branches and share a large core

genome where the distribution of antimicrobial resistance genes and virulence factors is similar. Besides, both clinical and environmental lineages present low susceptibility to antibiotics, supporting the idea that environmental strains are potential opportunistic resistant pathogens [131].

Although it is not considered as a highly virulent bacterium, it is an important opportunistic pathogen in clinical environments, being responsible for various infectious diseases. *S. maltophilia* infections can occur in the general population at the hospital setting, being most frequently associated with respiratory infections, followed by bloodstream infections, and, rarely, soft tissue and skin infections [132]. These diseases are usually associated with high mortality, particularly in immunocompromised individuals and in patients with underlying pathologies, as cystic fibrosis or cancer, as well as in those who have been under previous therapy with broad-spectrum antibiotics [129,132].

1.7.1. Antibiotic resistance mechanisms in *S. maltophilia*

The World Health Organization (WHO) lists *S. maltophilia* as one of the leading drug-resistant pathogens of greatest public health concern in hospitals worldwide [133]. This opportunistic pathogen shows an intrinsic low susceptibility to many antibiotics, including those that are commonly used to treat the infections that it causes. *S. maltophilia* isolates have demonstrated resistance against beta-lactams, cephalosporins, macrolides, fluoroquinolones, aminoglycosides, and carbapenems [134].

The low susceptibility displayed by this bacterium against antibiotics is due to the interplay of several resistance mechanisms [134,135]. Among them, *S. maltophilia* possesses two chromosomal-encoded inducible beta-lactamases, namely L1 and L2, which contribute to beta-lactams resistance. Expression of both enzymes is indirectly induced by beta-lactam antibiotics and controlled by the transcriptional regulator AmpR [136,137].

Regarding aminoglycoside resistance, *S. maltophilia* genome encodes several known and putative aminoglycoside-modifying enzymes, such as AAC(6')-Iz, an aminoglycoside acetyltransferase that confers resistance against amikacin, tobramycin, netilmicin and sisomicin [138]; APH(3')-IIc, an aminoglycoside phosphotransferase that contributes to kanamycin, neomycin, paromomycin and butirosin [139]; and a more recently

identified AAC(6')-Iak [140]. Further, susceptibility to aminoglycosides can vary in a transient manner depending on growth temperature as a consequence of outer membrane lipids alteration, being more susceptible to these antibiotics at 37 °C than at 30 °C [141].

Besides, *S. maltophilia* chromosome encodes the SmQnr protein, which contributes to low-level intrinsic resistance to quinolones [142]. Since *qnr* genes are usually plasmid-associated, it has been proposed that *S. maltophilia* isolates that harbour Sm*qnr* genes may act as a reservoir for the potential dissemination of these quinolone resistance elements to other *Enterobacteriaceae* [143].

1.7.2. MDR efflux pumps

Major contributors to the *S. maltophilia* low susceptibility to antibiotics are MDR efflux pumps belonging to the RND family. Eight RND-type systems that are encoded in the *S. maltophila* genome have been identified: SmeABC, SmeDEF, SmeGH, SmeJK, SmeMN, SmeOP, SmeVWX, and SmeYZ (Table 1) [135]. Regulation of these RND efflux pumps occurs at different levels, making *S. maltophilia* a suitable model for the study of these resistance determinants. The expression of some of them is modulated by local regulators, such as SmeDEF efflux pump, which is down-regulated by the TetR-family SmeT repressor [46], or SmeVWX, whose expression is controlled by the LysR-type transcriptional regulator SmeRv [144]. In other cases, TCSs are the regulators of *S. maltophilia* efflux pumps, as in SmeYZ, whose expression is controlled by the SmeRySy system [145], or SmeABC, regulated by the SmeRS TCS [146].

As previously stated, RND efflux pumps can be contributors to intrinsic, acquired or transient resistance. In *S. maltophilia*, the role of six of them (SmeABC, SmeDEF, SmeGH, SmeIJK, SmeOP, SmeVWX, and SmeYZ) in intrinsic, acquired and/or transient resistance has been previously analysed (Table 1) [39,146-151]. For instance, *smeYZ* is constitutively expressed at a significant level, thus playing an important role in the intrinsic resistance to several antibiotics, including aminoglycosides [39]. Antibiotic resistance can be also achieved through the overexpression of these efflux systems, which can be accomplished by the acquisition of mutations usually in the regulatory elements that control their expression. In the case of *S. maltophilia*, the most prevalent cause of acquired resistance to quinolones is the overproduction of MDR efflux pumps, particularly SmeDEF and SmeVWX, associated with

mutations in their respective transcriptional regulators [47,152]. Regarding transient resistance, the role of the *S. maltophilia* RND efflux pumps has not been studied in detail and just SmeDEF efflux pump has been reported to contribute to transient antibiotic resistance [68,69]. Thus, overexpression of RND efflux pumps leading to a transient situation of antibiotic resistance can also be achieved by changes in the expression of these systems caused by particular physiological situations or as a response to effectors [77]. Knowing the inducers of *S. maltophilia* efflux pumps would help to characterize these systems, not only regarding their ecological or functional role, but also for predicting situations of transient antibiotic resistance induction.

Table 1. Overview of the identified *S. maltophilia* RND efflux pumps and their role in antibiotic resistance

Efflux Pump	Antibiotic Resistance	IR	AR	TR	Ref.
SmeABC	Aminoglycosides, beta-lactams, fluoroquinolones.	No	Yes	ND	146
SmeDEF	Tetracycline, chloramphenicol, macrolides, fluoroquinolones, sulfamethoxazole, trimethoprim, trimethoprim/sulfamethoxazole.	Yes	Yes	Yes	147,150, 153,154
SmeGH	Unknown	ND	Yes	ND	135,151
SmeIJK	Aminoglycosides, tetracycline, minocycline, ciprofloxacin, levofloxacin, leucomycin	Yes	Yes	ND	148,155
SmeMN	Unknown	ND	ND	ND	135
SmeOP	Nalidixic acid, doxycycline, aminoglycosides, macrolides	Yes	No	ND	149
SmeVWX	Quinolones, chloramphenicol, trimethoprim/sulfamethoxazole	No	Yes	ND	47,150,152
SmeYZ	Aminoglycosides, tetracycline, leucomycin, trimethoprim/sulfamethoxazole	Yes	Yes	ND	39

IR: intrinsic resistance; AR: acquired resistance; TR: transient resistance; ND: not determined

1.7.3. Therapeutic strategies for the treatment of *S. maltophilia* infections

The intrinsic low susceptibility of *S. maltophilia* against antibiotics limits the antimicrobial options and complicate the treatment of its infections. The combination trimethoprim/sulfamethoxazole (SXT) is currently the most effective drug of choice [156]. It can also be used in combination with other agents, such as ciprofloxacin [157]. Nevertheless, the use of this antimicrobial is limited due to intolerance, allergic reactions and resistance mechanisms, such as *sul1*, *sul2* and *dfrA* genes, or the expression of MDR efflux pumps [150,158-161]. Thus, alternative therapeutic options are required to combat *S. maltophilia* infections.

Among them, the use of ceftazidime, a third-generation cephalosporin, mainly in combination with other antimicrobial agents, has been proposed as a potential treatment, since many *S. maltophilia* clinical isolates are susceptible to this beta-lactam [156,162-164]. Despite the fact that *S. maltophilia* possesses a large set of antibiotic resistance genes, the potential mechanisms that could challenge a ceftazidime-based treatment, besides beta-lactamase overproduction [165], have not been sufficiently studied.

Tigecycline, which belongs to a new group of tetracyclines called glycylcyclines, also appears as a suitable option for the treatment of *S. maltophilia* severe infections, mostly those with a nosocomial origin [166], being also effective against SXT-resistant *S. maltophilia* isolates [167,168]. Although the mechanisms of acquisition of resistance to tigecycline have been described for other bacterial species, in *S. maltophilia* the resistance mechanisms against this antibiotic are not well established.

In the course of this PhD thesis, we have focused our attention on the study of the role of *S. maltophilia* RND efflux pumps in transient resistance to antibiotics through the identification of molecules and/or signals that induce their expression, an issue that is still hardly explored. In addition, we aim to elucidate new potential mechanisms involved in the acquisition of ceftazidime and tigecycline resistance, two of the antibiotics of choice that are currently under study for the treatment of *S. maltophilia* infections, after experimental evolution in the presence of these antibiotics, as well as the effects of the selected mutations on bacterial physiology aspects, such as fitness cost. Consequently, in this PhD thesis, we have addressed the following objectives.

OBJECTIVES

2. Objectives

I. Study of the role of RND efflux pumps in the inducible resistance to antibiotics of *S. maltophilia*

a. To develop YFP-based reporters of the expression of SmeVWX and SmeYZ efflux pumps.
b. To perform a screening of potential inducer compounds that trigger the expression of either *smeVWX* and/or *smeYZ*.
c. To analyse the effect of the identified inducer compounds on the transient antibiotic resistance of *S. maltophilia*.

II. Study of the acquisition of resistance during experimental evolution in *S. maltophilia*

a. To perform experimental evolution assays in the presence of the antibiotics ceftazidime and tigecycline.
b. To unveil the genetic mechanisms underlying the acquisition of resistance to both antibiotics.
c. To assess how the acquisition of resistance influences cross-resistance and collateral sensitivity against other antibiotics, and on fitness cost.

RESULTS

3. Results

The results of this PhD thesis have been published in peer-reviewed journals with the following titles:

I. Vitamin K_3 Induces the Expression of the *Stenotrophomonas maltophilia* SmeVWX Multidrug Efflux Pump.
II. Biolog Phenotype Microarray Is a Tool for the Identification of Multidrug Resistance Efflux Pump Inducers.
III. Involvement of the RND Efflux Pump Transporter SmeH in the Acquisition of Resistance to Ceftazidime in *Stenotrophomonas maltophilia*.
IV. Mechanisms and Phenotypic Consequences of Tigecycline Resistance Acquisition in *Stenotrophomonas maltophilia*.

All the mentioned papers are completely reported hereunder, along with a brief introduction and the corresponding supplementary information.

The PhD candidate contributed to the experimental design of each study, development of the experiments, interpretation of the results and writing of the manuscripts, together with the other authors.

Article I

Vitamin K_3 Induces the Expression of the *Stenotrophomonas maltophilia* SmeVWX Multidrug Efflux Pump

P. Blanco, F. Corona, M. B. Sánchez, J. L. Martínez

Antimicrobial Agents and Chemotherapy. 61:e02453-16 (2017)

Multidrug (MDR) efflux pumps are major contributors to *S. maltophilia* antibiotic resistance, being the resistance nodulation division (RND) family the best characterized. Although their expression is usually controlled by specific transcriptional regulators, some situations, as the presence of certain effectors in the environment or particular physiological conditions, can induce the expression of these efflux systems, which might lead to a situation of transient resistance to antibiotics that are substrates of such efflux pumps. Thus, the knowledge of the effectors (or situations) that are able to trigger the expression of these resistance determinants is fundamental, not only to predict situations of transient antibiotic resistance, but also to understand the functional role of *S. maltophilia* MDR efflux pumps besides antibiotics extrusion.

In the present work, we carried out a screening of potential inducer compounds of the *S. maltophilia* SmeVWX RND efflux pump, proved to contribute to the acquisition of resistance against quinolones, chloramphenicol and trimethoprim/sulfamethoxazole (SXT). Therefore, we developed a yellow fluorescent protein (YFP)-based sensor and tested a variety of compounds in order to measure the fluorescence given by the expression of YFP under the control of the *smeVWX* promoter. Among the tested compounds, we found vitamin K_3, or menadione, as an inducer of the expression of *smeVWX*. Besides, the presence of this compound, which is produced by plants and is present in some haemostatic drugs, renders *S. maltophilia* less susceptible to ofloxacin and chloramphenicol, two of the SmeVWX antibiotic substrates.

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Vitamin K_3 Induces the Expression of the *Stenotrophomonas maltophilia* SmeVWX Multidrug Efflux Pump

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ABSTRACT *Stenotrophomonas maltophilia* is an opportunistic pathogen with increasing prevalence, which is able to cause infections in immunocompromised patients or in those with a previous pathology. The treatment of the infections caused by this bacterium is often complicated due to the several intrinsic antibiotic resistance mechanisms that it presents. Multidrug efflux pumps are among the best-studied mechanisms of *S. maltophilia* antibiotic resistance. Some of these efflux pumps have a basal expression level but, in general, their expression is often low and only reaches high levels when the local regulator is mutated or bacteria are in the presence of an effector. In the current work, we have developed a yellow fluorescent protein (YFP)-based sensor with the aim to identify effectors able to trigger the expression of SmeVWX, an efflux pump that confers resistance to quinolones, chloramphenicol, and tetracycline when it is expressed at high levels. With this purpose in mind, we tested a variety of different compounds and analyzed the fluorescence signal given by the expression of YFP under the control of the *smeVWX* promoter. Among the tested compounds, vitamin K_3, which is a compound belonging to the 2-methyl-1,4-naphthoquinone family, is produced by plants in defense against infection, and has increasing importance in human therapy, was able to induce the expression of the SmeVWX efflux pump. In addition, a decrease in the susceptibility of *S. maltophilia* to ofloxacin and chloramphenicol was observed in the presence of vitamin K_3, in both wild-type and *smeW*-deficient strains.

KEYWORDS inducible resistance, SmeVWX, *Stenotrophomonas maltophilia*, biosensors, efflux pumps

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Stenotrophomonas maltophilia is an emerging multidrug-resistant opportunistic pathogen involved in an increased number of infections (1). Among these infections, we can highlight septicemia, urinary infections, endocarditis, and respiratory infections in immunocompromised patients and in those with cystic fibrosis (2). In general, clinical isolates of *S. maltophilia* present low susceptibilities to a wide range of antibiotics, including macrolides, β-lactams, cephalosporins, trimethoprim-sulfamethoxazole, tetracyclines, polymyxins, aminoglycosides, chloramphenicol, carbapenems, and fluoroquinolones, making the infections caused by this bacterium difficult to treat (1). This low susceptibility to antibiotics is associated with several intrinsic resistance elements, such as antibiotic-modifying enzymes, low membrane permeability, the quinolone resistance protein SmQnr, and multidrug resistance (MDR) efflux pumps (3, 4).

Eight MDR efflux pumps (SmeABC, SmeDEF, SmeGH, SmeIJK, SmeMN, SmeOP, SmeVWX, and SmeYZ) belonging to the resistance-nodulation-cell division (RND) family have been identified in *S. maltophilia* K279a (4). The roles of six of them (SmeABC, SmeDEF, SmeIJK, SmeOP, SmeVWX, and SmeYZ) in intrinsic and acquired resistance to antibiotics have been analyzed (5–14).

The expression of MDR efflux pumps is generally tightly downregulated by specific transcriptional regulators, likely because their overexpression might compromise bac-

terial physiology (15). However, transient higher expression levels can be reached due to some particular physiological situations, as a response to stress or in the presence of effectors, including host-produced anti-infective molecules, such as bile, cationic peptides, or fatty acids (16–18), as well as agents used for therapeutic purposes, some of which might be relevant in the course of an infection (19). Knowing these effectors can be useful for understanding the role of these MDR efflux pumps besides resistance to antibiotics, as well as for predicting situations of transient antibiotic resistance that may occur *in vivo* (20).

RND efflux pump substrates are very diverse and include antibiotics, biocides, bile salts, detergents, aromatic hydrocarbons, homoserine lactones, and dyes (21); however, the number of known inducers that can trigger their expression is lower in comparison (20). In the present study, we have carried out a screening in order to identify potential effectors of the *S. maltophilia* RND efflux pump SmeVWX, using a yellow fluorescent protein (YFP)-based sensor. The operon encoding this MDR efflux system is composed of a membrane fusion protein gene (*smeV*), an inner membrane transporter gene (*smeW*), and an outer membrane protein gene (*smeX*). *smeVWX* genes are coexpressed with two short-chain-dehydrogenase/reductase (SDR) genes, which are located upstream *smeV* (*smeU1*) and between *smeW* and *smeX* (*smeU2*). The operon is regulated by the LysR-type transcriptional regulator SmeRv (11). SmeVWX does not participate in *S. maltophilia* intrinsic resistance, likely because its expression levels are too low (11); nevertheless, it is known that this efflux pump contributes to the acquisition of resistance mediated by mutations in the transcriptional regulator SmeRv, leading to the overexpression of the efflux system (22). Further, quinolone-resistant clinical isolates overexpressing this efflux pump have been found, indicating that SmeVWX overexpression may have clinical relevance (12, 22).

Among the compounds tested in this study, we have found that vitamin K_3 (vitK3), also known as menadione (2-methylnaphtalene-1,4-dione), acts as an inducer and is likely a substrate of the SmeVWX efflux system. In addition to developing a biosensor for tracking inducers of the expression of SmeVWX and since vitK3 is present in some hemostatic drugs and is also becoming an important candidate for anticancer therapy, our study provides information about the potential implications that using vitK3 during the course of an infection caused by *S. maltophilia* might have.

RESULTS AND DISCUSSION

Reporter construction. Fluorescent protein-based sensors have been extensively used for different purposes due to their high reliability, sensitivity, and simplicity in operation (23). In the current work, a YFP-based reporter was constructed in order to identify potential inducers of the expression of the *S. maltophilia* SmeVWX efflux pump, which are unknown so far. To carry this out, a DNA fragment containing the promoter sequence of *smeVWX* was amplified and cloned into the pSEVA237Y plasmid, which harbors the yellow fluorescent protein (YFP) (24), giving rise to the pPBT04 plasmid, as described in Materials and Methods. This allows the quantification of the expression of *smeVWX* through the fluorescence given by YFP. Using tripartite conjugation, the plasmid pPBT04 was introduced in *S. maltophilia* D457, with the resultant strain dubbed PBT02, and in *S. maltophilia* MBS287, the resultant strain was dubbed PBT06. The MBS287 strain, a mutant derived from the wild-type *S. maltophilia* D457 strain, constitutively expresses high levels of *smeVWX* due to a mutation (Gly266Asp) in the gene encoding its local regulator SmeRv. This strain can be used as a control for measuring the expression levels of *smeVWX* when the efflux pump is overexpressed (22). To test the *smeVWX* sensor, the fluorescence levels given by the PBT02 and PBT06 strains and the growth of both strains were measured for 18 h. As mentioned above, the expression levels of SmeVWX are very low; consequently, it is expected that the fluorescence given by the expression of YFP under the control of the *smeVWX* promoter in the PBT02 strain is lower than the one obtained in the overexpressing derivative strain PBT06. As shown in Fig. 1, higher levels of fluorescence given by the YFP are

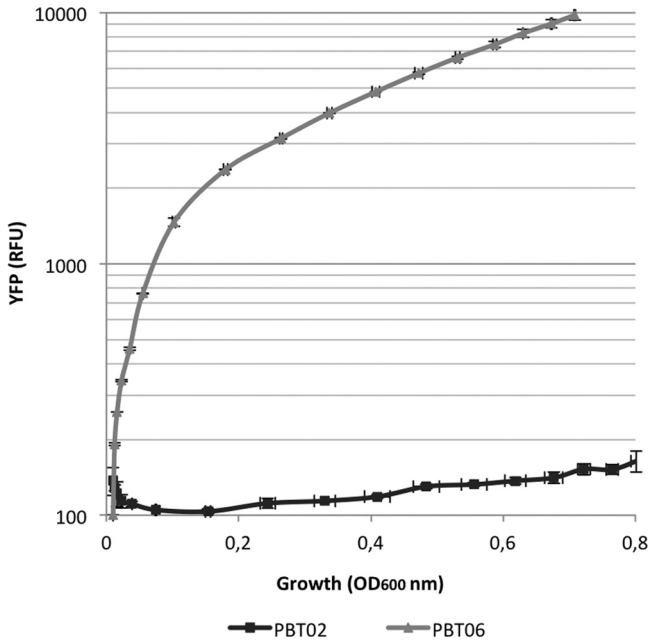

FIG 1 Fluorescence levels and growth of the sensor strains PBT02 and PBT06. The fluorescence of both strains was measured using a plate reader for 18 h in LB medium. The YFP levels given by the expression of *smeVWX* in PBT06 are higher than in the D457-derivative strain PBT02, since it overexpresses the efflux pump due to a mutation in the regulator SmeRv. There are not significant differences in the growth of both strains. Error bars indicate standard deviations of the results from three independent replicates. RFU, relative fluorescence units.

obtained in the case of the PBT06 strain than with PBT02 during growth, validating the *smeVWX* sensor developed in this work.

Vitamin K_3 induces *smeVWX* expression. Multidrug efflux pumps are relevant elements in the development of antibiotic resistance in bacterial populations. Expression of these determinants is usually repressed by specific regulators encoded by genes located upstream of the operons that contain the structural genes for these efflux pumps (19). In the opportunistic pathogen *S. maltophilia*, some of these RND multidrug efflux pumps, such as SmeDEF, SmeIJK, SmeOP, and SmeYZ, contribute to bacterial intrinsic resistance (4, 6, 9, 14), while others, such as SmeVWX, have very low levels of expression and its overexpression alone leads to acquired resistance (11, 22). In addition to genetic alterations, resistance can also be achieved by the presence of effectors or conditions that trigger the expression of multidrug efflux pumps, leading to the acquisition of transient phenotypic antibiotic resistance (25–27). A wide range of MDR efflux pump substrates are known. However, the number of known effectors that regulate their expression is lower in comparison (28). For instance, the AcrAB-TolC system in *Escherichia coli* can be induced by bile salts (29), and in *Pseudomonas aeruginosa*, the MexXY-OprM system is induced in response to antibiotics that target the ribosome and under oxidative stress conditions (30, 31). In the case of *S. maltophilia*, the SmeDEF efflux pump is induced by triclosan and some plant-produced compounds, which bind its local repressor SmeT, so that *smeDEF* transcription becomes activated (26, 32). To date, it is not known if there is any compound able to induce the expression of the SmeVWX efflux pump; consequently, we carried out a screening of such potential effectors in *S. maltophilia* PBT02 using compounds belonging to different categories, including antibiotics, compounds that produce oxidative stress, chelating agents, biocides, etc. The concentrations were chosen taking into consideration the MIC values of each compound.

None of the tested compounds gave a clear increase in fluorescence levels, except vitK3, which caused an increment in the fluorescence obtained by YFP

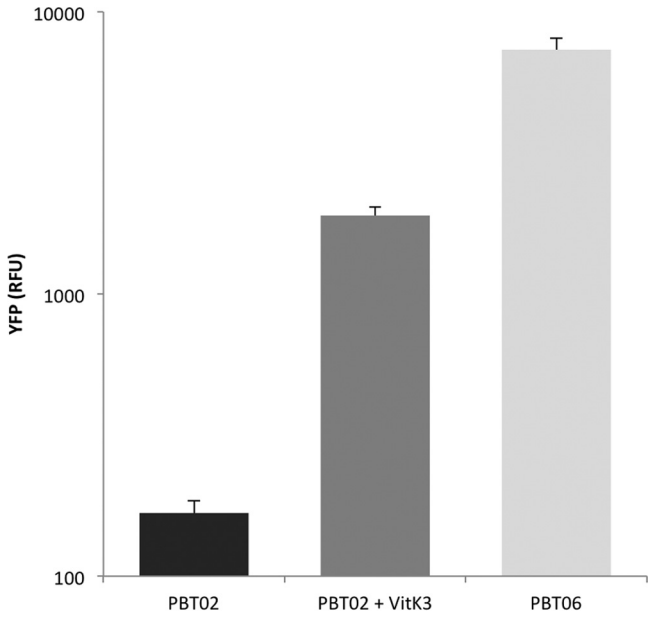

FIG 2 Effect of vitK3 on *smeVWX* expression. The fluorescence levels obtained by *smeVWX* expression when OD_{600} of 0.6 is reached in PBT02 strain, PBT02 strain after incubation with 1 mM vitK3, and PBT06 strain are shown. vitK3 increases YFP expression in comparison with the untreated strain, although the level reached is lower than in PBT06. Error bars indicate standard deviations of the results from three independent replicates.

expression under control of the *smeVWX* promoter. The fluorescence levels were measured in both PBT02 and PBT06. As shown in Fig. 2, the fluorescence levels given by PBT02, when cells reached exponential phase (optical density at 600 nm [OD_{600}], 0.6), are higher in the presence of 1 mM vitK3 than those obtained in the absence of any compound. To analyze whether or not the effect of vitK3 was specific and determine the minimal concentrations that may trigger *smeVWX* induction, a more detailed analysis was performed using as potential inducers vitK3, its structural analogues vitamin K_2 (vitK2) and plumbagin, and the generator of oxidative stress, tert-butyl hydroperoxide. As shown in Fig. 3, vitK3 remains the best *smeVWX* inducer, even at concentrations as low as 4 μM, whereas a modest induction in the presence of vitK2 and plumbagin can be seen, and tert-butyl hydroperoxide does not induce the expression of *smeVWX*; this indicates that oxidative stress is not the cause (at least the unique cause) of the induction of *smeVWX* expression in the presence of vitK3.

In order to further address whether or not vitK3 induces the expression of *smeVWX*, *smeV* mRNA levels were measured by real-time reverse transcription-PCR (RT-PCR) in the presence and absence of vitK3. As shown in Fig. 4, the expression levels of *smeV* are increased by 105-fold in the presence of vitK3 in the wild-type strain D457 in comparison with those levels obtained without this compound. In *S. maltophilia* MBS287, where *smeVWX* is overexpressed, the *smeV* levels are increased by 286-fold in comparison with those of the wild-type strain D457. These data show that vitK3 increases the expression of *smeVWX* mRNA, a feature in agreement with the results obtained using the above-described fluorescent reporter, indicating that the sensor is valid for the detection of inducer compounds.

Vitamin K_3 might be extruded by SmeVWX efflux pump. As described above, vitK3 is able to induce *smeVWX* expression, and it is possible that this compound is a substrate of the efflux pump. To test this possibility, a D457-derivative mutant with a partial deletion in *smeW* (MBS704) was constructed by homologous recombination, as described in Materials and Methods. Both strains, the wild type (D457) and the *smeW* mutant (MBS704), were grown in the presence and absence of vitK3 (1 mM) for 20 h at

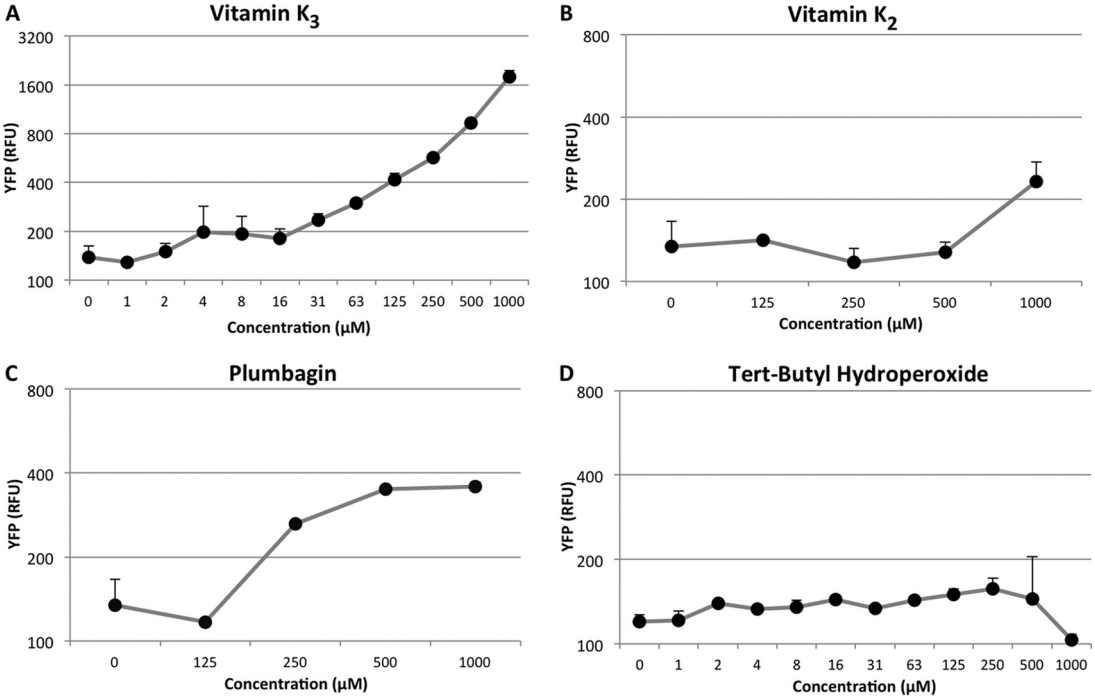

FIG 3 Effects of different compounds on *smeVWX* expression. Fluorescence levels for: vitamin K_3 (A), vitamin K_2 (B), plumbagin (C), and tert-butyl hydroperoxide (D) given by the expression of *smeVWX* in PBT02 strain when OD_{600} of 0.6 is reached at several concentrations. Plumbagin and vitamin K_2 slightly increase the YFP levels in comparison with vitamin K_3 (A to C); tert-butyl hydroperoxide does not cause any changes (D). Error bars indicate standard deviations of the results from three independent replicates.

37°C, and the optical density at 600 nm was measured every 10 min using a Tecan Infinite 200 plate reader (Tecan). As shown in Fig. 5, in the absence of vitK3, the two strains exhibit similar growth; however, when vitK3 is added to the medium, the MBS704 strain growth is impaired in comparison with D457. This result suggests that SmeVWX extrudes vitK3, and in the absence of this efflux pump, this compound inhibits *S. maltophilia* growth.

vitK3 is an analogue of vitamins K_1 and K_2, with all of them belonging to the 2-methyl-1,4-naphthoquinone family (33, 34). vitK3 is also known as menadione, a compound that has been isolated from fungi and phanerogams (35), where it was first studied as a plant regulator (36). The function of menadione in the defense against plant pathogens has been shown in several studies (37–39). This contribution can be carried out in two ways: first, vitK3 is able to increase the activity of the H^+-ATPase in plant cells due to its redox properties, contributing to the immune response against phytopathogens (40, 41); second, vitK3 itself is toxic for bacteria as a result of its capability of generating reactive oxygen species (ROS), elevating the intracellular production of O_2^- and H_2O_2 (42).

S. maltophilia is an ubiquitous bacterium which has been isolated from several sources, including those associated with the plant rhizosphere (1, 43). One of the roles of MDR efflux pumps is the prevention of the accumulation of toxic compounds inside the cell by extruding them (44). Since *S. maltophilia* is a rhizosphere-related bacterium, the SmeVWX efflux pump is likely involved in nature in the detoxification of vitK3 and its analogues, which might be produced by plants during *S. maltophilia* root colonization.

Vitamin K_3 decreases *S. maltophilia* antibiotic susceptibility even in the absence of *smeW*. It is known that the SmeVWX efflux pump is able to extrude quinolones and chloramphenicol, and its overexpression causes resistance to these antibiotics (11, 12). Since vitK3 is able to induce *smeVWX* expression, it is possible that the susceptibility to such antibiotics decreases in the presence of this agent. To analyze this hypothesis, a susceptibility assay using either an ofloxacin disc (5 μg) or a

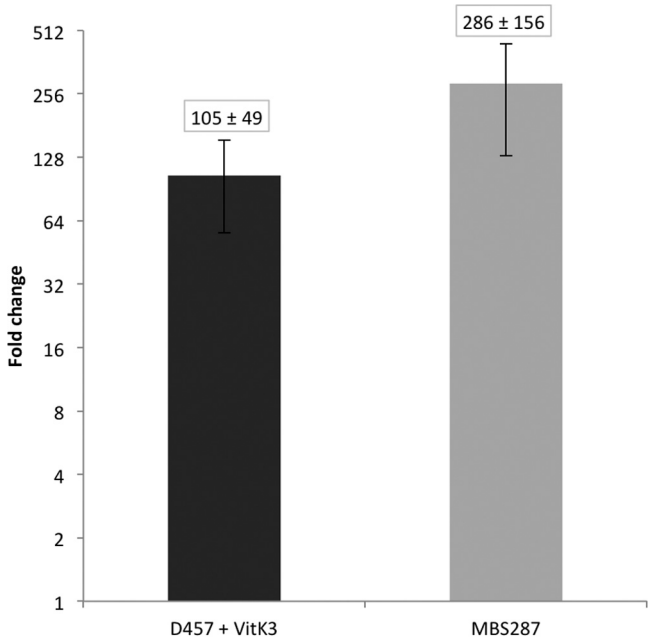

FIG 4 vitK3 increases *smeV* mRNA levels. *smeV* mRNA levels were measured by real-time RT-PCR in the presence of vitK3 in the wild-type D457 strain and in the MBS287 strain, which overexpresses *smeVWX*. The data show that expression of *smeV* is induced by vitK3. Fold changes were estimated with respect to the value given by strain D457 in the absence of vitK3. Error bars indicate standard deviations of the results from three independent experiments.

chloramphenicol disc (30 μg) placed next to a vitK3-containing disc (2 μmol) was carried out. The inhibition halos around the antibiotic discs present deformations in the region nearby the vitK3 disc (not shown), indicating that this compound decreases *S. maltophilia* susceptibility to antibiotics. To further confirm that vitK3 decreases susceptibility to antibiotics, a checkerboard assay was performed. A 96-well microtiter plate containing serial concentrations of either ofloxacin or chloramphenicol (also a substrate of SmeVWX) in combination with vitK3 was inoculated with cultures of *S. maltophilia* D457 or the *smeW*-defective mutant MBS704 at an OD_{600} of 0.01. After 24 h of incubation at 37°C, the OD_{600} was measured in each well. The fractional inhibitory concentration (FIC) values are shown in Table 1. Noteworthy, the effect of vitK3 was antagonistic in all cases, independently of the type of the antibiotic used and of the presence or the absence of *smeW*. This indicates that the effect of vitK3 on *S. maltophilia* goes beyond the induction of *smeVWX*, a topic that is currently under study in our laboratory.

Concluding remarks. Resistance to antibiotics has become an increasing problem in public health, with MDR efflux pumps being relevant elements in the development of this resistance. As mentioned above, MDR efflux pump expression is usually repressed by specific transcriptional regulators (15). Expression of MDR efflux pumps is likely induced under physiological conditions when their activity is required, and bacteria can determine this need by detecting growing conditions or compounds in the environment that act as effectors. However, antibiotics are not usually good inducers of efflux pumps, whereas nonantibiotic compounds can induce such expression (21).

In this study, we have developed a fluorescent biosensor useful for detecting inducers of the *S. maltophilia* SmeVWX MDR efflux pump. Using this biosensor, we have determined that vitK3 (a potential substrate of this efflux pump involved in the defense of plants against pathogens) is able to induce the expression of the *S. maltophilia* SmeVWX efflux pump. Vitamin K is an essential nutrient which acts as a cofactor in the production of some factors of blood coagulation in mammals, such as factors II (prothrombin), VII (proconvertin), IX, and X (33). vitK3 has improved antihemorrhagic

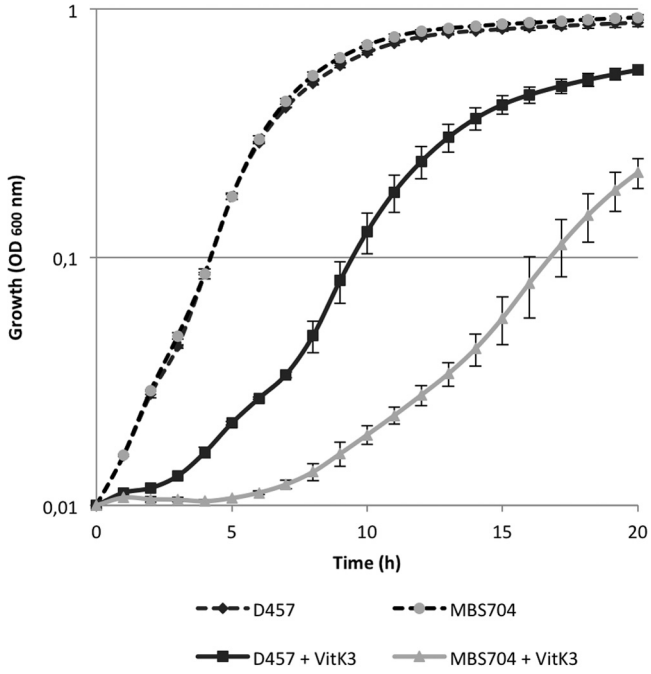

FIG 5 vitK3 might be extruded by the SmeVWX multidrug efflux pump. The growth in the presence and absence of 1 mM vitK3 in D457 strain and MBS704 (Δ*smeW*) was measured. In the presence of vitK3, the *smeW* mutant presents a diminished growth in comparison with the wild-type strain D457, suggesting that vitK3 might be extruded by the SmeVWX MDR efflux pump. Error bars indicate standard deviations of the results from three independent replicates.

activity compared with the natural vitamin K, so it is used as an agent of choice for the treatment of vitamin K deficiency, hemorrhagic diathesis, and hypoprothrombinemia, prophylactically before and after surgery to prevent bleeding, and it is administered to newborns with low levels of prothrombin to prevent hemorrhagic diseases (45). Besides, vitK3 is gaining importance as an anticancer agent because of its cytotoxic effect against cancer cells (34, 46). Recent work has shown that the plasma concentration of menadione upon the administration of 10 mg of menadiol sodium diphosphate to healthy subjects can reach a peak of 3 μM (47), within the concentration range at which we begin to observe induction of *smeVWX* expression.

S. maltophilia mainly causes nosocomial infections (48), although community-acquired infections can also occur (49). The population at risk is mostly composed of immunocompromised hosts, including intensive care unit (ICU) patients, patients with dialysis catheters, hematological diseases, and cystic fibrosis, and those treated with a wide spectrum of antibiotics (50–53). Although our results indicate that vitK3 might induce the expression of the *smeVWX* efflux pump even at low concentrations and antagonizes the effects of ofloxacin and chloramphenicol against *S. maltophilia*, both in the presence and in the absence of this efflux pump, the clinical significance of these findings remains to be clearly established.

In addition, the fact that vitK3 is produced by plants and that *S. maltophilia* is a common plant inhabitant (54) strongly suggests that SmeVWX might be involved in

TABLE 1 FIC values of vitK3 in combination with either chloramphenicol or ofloxacin[a]

	Chloramphenicol + vitK3					Ofloxacin + vitK3				
Strain	C_{vitK3}	MIC_{vitK3}	C_{chlor}	MIC_{chlor}	ΣFIC	C_{vitK3}	MIC_{vitK3}	C_{oflox}	MIC_{oflox}	ΣFIC
D457	2.56	2.56	16	16	2	5.12	5.12	6	4	2.5
MBS704	5.12	2.56	16	12	3	2.56	2.56	6	3	3

[a]See "FIC index analysis" in Materials and Methods for details on the terms used here. chlor, chloramphenicol; oflox, ofloxacin.

TABLE 2 Bacterial strains and plasmids

Strain or plasmid	Description[a]	Source or reference
Bacterial strains		
E. coli		
OmniMAX	Strain used in transformation; F′ {*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*(Tet^r)} *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80(*lacZ*)ΔM15 Δ(*lacZYA-argF*) *U169 endA1 supE44 thi-1 gyrA96 relA1 deoR tonA panD*	Invitrogen, Life Technologies
TGI	Strain used in transformation; *supE thi-1* Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)5 (r_K^- m_K^-) [F′ *traD36 proAB*$^+$ *lacI*^{q} *Z*ΔM15]	61
CC118λ*pir*	Donor cell in conjugation; strain CC118 lysogenized with λ *pir* phage (Tc′) Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE* (Am) *recA1*	56
1047 (pRK2013)	Helper cell in conjugation harboring pRK2013 (Kan^r) plasmid	62
S. maltophilia		
D457	Clinical strain	63
MBS287	D457-derived mutant (SmeRv G266N) overexpressing SmeVWX efflux pump	22
MBS704	D457 Δ*smeW*	This work
PBT02	D457 with pPBT04 plasmid	This work
PBT06	MBS287 with pPBT04 plasmid	This work
Plasmids		
pGEM-T Easy vector	Cloning vector, Amp^r	Promega
pSEVA237Y	Plasmid containing YFP; replication origin pBBR1; Kan^r	24
pPBT02	pGEMT-derived plasmid containing *smeVWX* promoter region	This work
pPBT04	pSEVA237Y-derived plasmid containing *smeVWX* promoter region	This work
pEX18Tc	Gene replacement vector; *sacB*, Tc^r	23
pBS51	pGEMT-derived plasmid containing the 5′ and 3′ regions of *smeW* gene	This work
pBS52	pEX18Tc-derived plasmid containing the 5′ and 3′ regions of *smeW* gene	This work

[a]Tet^r, tetracycline resistance; Kan^r, kanamycin resistance; Amp^r, ampicillin resistance.

bacterium-plant interactions in nature, a functional role that has been already demonstrated for the *S. maltophilia* SmeDEF efflux pump (32).

MATERIALS AND METHODS

Bacterial strains and growing conditions. All bacterial strains and plasmids used in this study are listed in Table 2. Cells were grown in LB medium at 37°C, unless otherwise stated. When required, the following antibiotics were added: ampicillin (Ap; 100 μg/ml) for *Escherichia coli* harboring the pGEM-T Easy vector and the pPBT02 and pBS51 plasmids, and kanamycin (Km; 50 μg/ml and 500 μg/ml) for *E. coli* and *S. maltophilia* D457, respectively, for pSEVA237Y and pPBT04 plasmid selection. Km at 50 μg/ml was added to LB liquid medium to maintain pSEVA237Y and pPBT04 plasmids in both *E. coli* and *S. maltophilia*; tetracycline (Tc; 10 μg/ml) was added in the case of *E. coli* harboring pEx18Tc and pBS52 plasmids. Tc at 10 μg/ml and imipenem (Imp; 20 μg/ml) were added for the selection of *S. maltophilia* D457 exconjugants. Medium was supplemented with 80 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for the detection of β-galactosidase production.

Reporter construction. *S. maltophilia* D457 genomic DNA was extracted according to the Gnome DNA kit protocol (MP Biomedicals). The 384-bp region between the *smeRv* gene (SMD_1762) and the *smeU1* gene (SMD_1763), which contains the *smeVWX* promoter region, was amplified using the FailSafe PCR system (Epicentre) with primers SmeVWX_F (5′-GAATTCGATCCTGGACGTCG-3′, EcoRI site underlined) and SmeVWX_R (5′-AAGCTTGACATTTCCTCCCAAATC-3′, HindIII site underlined). The thermocycler was programmed for 25 cycles of 94°C for 30 s of denaturation, 56°C for 30 s of annealing, and 72°C for a 36-s extension, with an initial denaturation at 94°C for 5 min and a final extension at 72°C for 7 min. The PCR product was ligated into pGEM-T Easy vector (Promega), according to the manufacturer's instructions, obtaining the pPBT02 plasmid, which was introduced by transformation into *E. coli* OmniMAX (Invitrogen). The construction was verified by DNA sequencing. The pPBT02 plasmid was extracted with the QIAprep Spin miniprep kit 250 (Qiagen), according to the manufacturer's instructions, and digested with EcoRI and HindIII restriction enzymes (New England BioLabs). The product corresponding to the *smeVWX* promoter region was purified with the purification kit (GE Healthcare) from a 1% agarose gel and cloned into pSEVA237Y using the same restriction enzymes and the T4 DNA ligase (New England BioLabs). The obtained plasmid, pPBT04, was introduced by transformation in *E. coli* OmniMAX (Invitrogen) competent cells. The presence of the plasmid was confirmed by PCR, as described above, with primers pSEVA227Y_F (5′-GCGGATAACAATTTCACACA-3′) and pSEVA227Y_R (5′-TTGCTCACCATATGTTTTTCC-3′).

The pPBT04 plasmid was introduced by transformation in *E. coli* CC118λ*pir*. Afterwards, the plasmid was introduced into the *S. maltophilia* D457 and MBS287 strains by tripartite matting using the strains *E. coli* CC118λ*pir* (donor cell), *E. coli* 1047/pRK2013 (helper cell), and *S. maltophilia* (receptor cell), in a 4:2:1 proportion (receptor:donor:helper). Kanamycin (500 μg/ml) was added for selecting the *S. maltophilia* clones containing the pPBT04 plasmid, and imipenem (20 μg/ml) was added for eliminating the *E. coli*

strains. For plasmid confirmation, PCR was performed as described above with primers pSEVA227Y_F and pSEVA227Y_R.

Deletion of the *smeW* gene. An *S. maltophilia* D457 mutant with a partial deletion of *smeW* gene was generated by homologous recombination. A fragment homologous to both the 5′ end (498 bp) and the 3′ end (513 bp) of the *smeW* gene was obtained by overlapping PCR, using the PCR master mix (Promega). In the first PCR, the primers SmeWA (5′-CGGGATCCTTAGCTGCCGGCGCCAG-3′, BamHI site underlined) and SmeWB (5′-CAGGATCTTCTGCGTAGTCA-3′) for the 5′ end and SmeWC (5′-CAGGATCTT CTGCGTAGTCA-3′) and SmeWD (5′-CCCAAGCTTGATGCATGCCTTGTGG3′, HindIII site underlined) for the 3′ end were used. The PCR products were purified with the QIAquick PCR purification kit (Qiagen), according to the manufacturer's instructions, and used as the template for a second PCR with SmeWA and SmeWD primers. The PCR product, purified from an agarose gel with the QIAquick gel extraction kit (Qiagen), was cloned in pGEM-T Easy vector (Promega), generating pBS51 plasmid, which was introduced in *E. coli* TG1. The right sequence was confirmed by sequencing, and the fragment containing the 5′ and 3′ ends of the *smeW* gene was cloned into the suicide vector pEX18Tc (55) using the BamHI-HindIII sites, generating the pBS52 plasmid. This plasmid was introduced into *E. coli* CC118λpir and mobilized afterwards into *S. maltophilia* D457 by tripartite conjugation (56). The exconjugants containing pBS52 were selected on LB agar containing 10 mg/ml tetracycline and 20 mg/ml imipenem. Tetracycline-resistant colonies were streaked onto 10% sucrose-LB agar to select double recombinants with the partial deletion of the *smeW* gene. *S. maltophilia* D457 Δ*smeW* (MBS704) was confirmed by PCR, with external (SmeWA/SmeWD) and internal (SmeW5 [5′-GAACCGTTGCCGAACAGC-3′]/SmeW6 [5′-GACAGGCCTTCCT CGATG-3′]) primers to the *smeW* gene.

Screening of potential inducers of the expression of *smeVWX*. Different compounds from different categories were used in this assay at serial concentrations. Among them were the antibiotics erythromycin (512, 256, and 128 μg/ml), gentamicin (32, 16, and 8 μg/ml), co-trimoxazole (trimethoprim-sulfamethoxazole, 1:5) (1, 0.5, and 0.25 μg/ml), chloramphenicol (16, 8, and 4 μg/ml), tobramycin (512, 256, and 128 μg/ml), ofloxacin (4, 2, and 1 μg/ml), kanamycin (512, 256, and 128 μg/ml), tetracycline (4, 2, and 1 μg/ml), polymyxin B (2, 1, and 0.5 μg/ml), and colistin (24, 12, and 6 μg/ml); heavy metals $ZnSO_4$ (5, 2.5, and 1.25 mM), $CuSO_4$ (5, 2.5, and 1.25 mM), $CdSO_4$ (5, 2.5, and 1.25 mM), $CoSO_4$ (5, 2.5, and 1.25 mM), and $FeCl_3$ (5, 2.5, and 1.25 mM); oxidative stress compounds paraquat (5, 2.5, and 1.25 mM), vitamin K_3 (2, 1, and 0.5 mM), and tert-butyl hydroperoxide (1, 0.5, and 0.25 mM); biocides triclosan (10, 5, and 2.5 μg/ml) and hexachlorophene (100, 50, and 25 μg/ml); plant-produced flavonoids phloretin (10, 5, and 2.5 μg/ml), quercetin (10, 5, and 2.5 μg/ml), and genistein (10, 5, and 2.5 μg/ml); detergents SDS (200, 100, and 50 mM) and Tween 20 (200, 100, and 50 mM); chelating agents EDTA (2, 1, and 0.5 mM) and EGTA (2, 1, and 0.5 mM); the analgesic metamizol (10, 5, and 2.5 mg/ml); and the inhibitor of oxidative phosphorylation carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP; 20, 10, and 5 μM). The compounds vitamin K_3, vitamin K_2, plumbagin, and tert-butyl hydroperoxide were studied in more detail using a wider range of concentrations.

The stock solutions of the different compounds were diluted in LB medium to obtain the required concentrations. The assay was performed in Corning Costar 96-well black clear-bottom plates (Corning Incorporated). Ten microliters of cell culture was inoculated in 140 μl of medium in each well to a final OD_{600} of 0.01. Bacteria were grown at 37°C for 18 h, and growth (OD_{600}) and fluorescence were measured every 10 min (although data are represented on an hourly basis) using a Tecan Infinite 200 plate reader (Tecan) set with an excitation wavelength at 508 nm and emission wavelength at 540 nm for YFP detection.

Assays of induction of antibiotic resistance. Two hundred microliters of *S. maltophilia* D457 culture at an OD_{600} of 0.01 was seeded in an agar-LB medium plate. A 9-mm sterile disc (Macherey-Nagel) was placed in the plate with vitK3 (2 μmol). An ofloxacin (5 μg) or a chloramphenicol disc (30 μg) (Oxoid) was placed 18 mm from the vitK3 disc. The plate was incubated for 24 h at 37°C.

The effect of vitK3 on the susceptibility of *S. maltophilia* to ofloxacin or chloramphenicol was analyzed using the checkerboard technique in a 96-well microtiter plate, where 10 μl of bacterial culture of D457 or MBS704 strain was added to each well to a final OD_{600} of 0.01. Eleven vitK3 concentrations were combined with 7 ofloxacin or chloramphenicol concentrations to carry out this assay. The plates were incubated at 37°C, and the OD_{600} was recorded after 24 h using a Tecan Infinite 200 plate reader (Tecan).

FIC index analysis. The fractional inhibitory concentration (FIC) index (57) was determined for each strain in order to define if there is some interaction between the antibiotics and vitK3. The sum of the FICs is defined as $\Sigma FIC = FIC_A + FIC_B = (C_A/MIC_A) + (C_B/MIC_B)$, where A is vitK3 and B is ofloxacin or chloramphenicol. The MIC is defined as the minimum inhibitory concentration of the compounds alone, and C is defined as the MIC of the compounds in combination. Synergy is defined as an FIC of ≤0.5, an FIC value between 0.5 and 2 is considered indifferent, and antagonism is defined as an FIC value of ≤2.

RNA preparation and real-time RT-PCR. Two flasks containing 20 ml of LB medium were inoculated with an overnight culture of *S. maltophilia* D457 to a final OD_{600} of 0.01 and were incubated until exponential phase was reached (OD_{600}, 0.6). This step was repeated, inoculating two new flasks with culture from the previous ones. When the culture reached an OD_{600} of 0.6, vitK3 (1 mM) was added to one of the flasks and cells were incubated for a further 30 min. Ten milliliters of each culture was centrifuged at 8,000 rpm and 4°C for 20 min. Five hundred seventy microliters of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and 30 μl of lysozyme (Sigma) from a 20 mg/ml stock, for a final concentration of 1 mg/ml, were added to each sample. Afterwards, the samples were mixed by vortexing for 10 s and were incubated at room temperature for at least 10 min with regular vortexing. A volume of 2,100 μl of buffer RLT (Qiagen) was added, and samples were sonicated twice for 5 to 10 s (constant

frequency, 0.45 Hz). Then, 1,410 μl of ethanol was added and the protocol continued using the RNeasy minikit (Qiagen), according to the manufacturer's instructions. In order to eliminate any residual DNA, a DNase I (Qiagen) treatment was carried out, according to the manufacturer's instructions. A second treatment was performed using Turbo DNA-free (Ambion). RNA integrity was verified on a 1% agarose gel, and the absence of DNA was confirmed by PCR using primers *sme*27 and *sme*48, which amplify a 347-bp fragment belonging to the *smeT* gene from strain D457 (58). cDNA was obtained from 10 μg of RNA using the High-Capacity cDNA reverse transcription kit (Applied Biosystems). Real-time RT-PCR was performed using the Power SYBR green PCR master mix (Applied Biosystems) in the ABI Prism 7500 real-time system (Applied Biosystems). The first denaturation step, 95°C for 10 min, was followed by 40 temperature cycles (95°C for 15 s, 60°C for 1 min) for amplification and quantification. Fifty nanograms of cDNA was used in each reaction. Primers SmeV-RT.fw and SmeV-RT.rv, which amplify the *smeV* gene (22), were used at 400 nM. Primers FtsZ1 and FtsZ2 were used to amplify the housekeeping gene *ftsZ* (22). Differences in the relative amounts of mRNA were determined according to the $2^{-\Delta\Delta CT}$ method (59, 60). In all cases, the mean values for relative mRNA expression were obtained from three independent experiments.

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Article II

Biolog Phenotype Microarray Is a Tool for the Identification of Multidrug Resistance Efflux Pump Inducers

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S. maltophilia RND efflux pumps present diverse regulation patterns, which causes that each efflux pump contributes differently to resistance. While SmeVWX, regulated by the LysR-type regulator SmeRv, is expressed at very low levels at laboratory conditions and hence, does not contribute to the bacterial intrinsic resistance, the SmeYZ efflux is controlled by the two-component system (TCS) SmeRySy and is constitutively expressed, being an intrinsic resistance determinant against aminoglycosides. As discussed in Article I, an increased expression of these efflux systems can be induced by environmental signals/cues.

With the aim of extending our knowledge about the effectors that are able to trigger the expression of the *S. maltophilia* efflux pumps, we developed a YFP-based sensor corresponding to the SmeYZ efflux pump. In this case, we used the Biolog phenotype microarrays as a high-throughput methodology for the identification of inducer molecules of *smeYZ*, as well as for searching new potential inducers of *smeVWX*. Among all the tested compounds, we found that iodoacetate, clioquinol, and sodium selenite are able to induce *smeVWX*, while boric acid and the antibiotics erythromycin, chloramphenicol, and lincomycin, trigger *smeYZ* expression. The mechanisms of action of the identified compounds suggest that *smeVWX* induction might be associated with the thiol-reactivity of the inducer molecules, while ribosome-stalling stress could be the signal that triggers *smeYZ* expression. We also analysed the effect of the exposure to the inducers sodium selenite and lincomycin on the transient resistance to antibiotics, finding that both molecules are able to promote transient resistance to ofloxacin (substrate of SmeVWX) and amikacin (substrate of SmeYZ), respectively.

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MECHANISMS OF RESISTANCE

Biolog Phenotype Microarray Is a Tool for the Identification of Multidrug Resistance Efflux Pump Inducers

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ABSTRACT Multidrug resistance efflux pumps frequently present low levels of basal expression. However, antibiotic-resistant mutants that overexpress these resistance determinants are selected during infection. In addition, increased expression of efflux pumps can be induced by environmental signals/cues, which can lead to situations of transient antibiotic resistance. In this study, we have applied a novel high-throughput methodology in order to identify inducers able to trigger the expression of the *Stenotrophomonas maltophilia* SmeVWX and SmeYZ efflux pumps. To that end, bioreporters in which the expression of the yellow fluorescent protein (YFP) is linked to the activity of either *smeVWX* or *smeYZ* promoters were developed and used for the screening of potential inducers of the expression of these efflux pumps using Biolog phenotype microarrays. YFP production was also measured by flow cytometry, and the levels of expression of *smeV* and *smeY* in the presence of a set of selected compounds were also determined by real-time reverse transcription-PCR (RT-PCR). The expression of *smeVWX* was induced by iodoacetate, clioquinol, and selenite, while boric acid, erythromycin, chloramphenicol, and lincomycin triggered *smeYZ* expression. The susceptibility to antibiotics that are known substrates of the efflux pumps decreased in the presence of the inducers. However, the analyzed multidrug efflux systems did not contribute to *S. maltophilia* resistance to the studied inducers. To sum up, the use of fluorescent bioreporters in combination with Biolog plates is a valuable tool for identifying inducers of the expression of bacterial multidrug resistance efflux pumps, and likely of other bacterial systems whose expression is regulated in response to signals/cues.

KEYWORDS antibiotic resistance, efflux pumps, induction of resistance, phenotype microarrays, phenotypic resistance, *Stenotrophomonas maltophilia*

Multidrug resistance (MDR) efflux pumps constitute a group of antibiotic resistance determinants able to reduce the activity of antimicrobial agents through their active transport outside the cell (1–3). Expression of efflux pumps is usually tightly downregulated at several levels and frequently involves the participation of global and local transcriptional regulators (4). Depending on their basal level of expression, some efflux pumps contribute to intrinsic resistance, while the contribution of others to this phenotype is low (3, 5, 6). In addition to their contribution to intrinsic resistance to antimicrobials, MDR-overexpressing mutants presenting decreased susceptibility (cross-resistance) to different clinically useful antibiotics (7) are isolated from infected patients (8) and selected *in vitro* upon antibiotic selective pressure (9). Five different families of MDR efflux pumps have been described so far (6). Among them, the resistance-nodulation-division (RND) MDR efflux pumps family stands as the most relevant one in Gram-negative bacteria (10, 11).

Stenotrophomonas maltophilia is an opportunistic pathogen, with environmental origin (12), which produces nosocomial infections as well as chronic infections in cystic fibrosis patients (13, 14). *S. maltophilia* is considered a prototype of intrinsically resistant

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pathogens (15). The characteristic low susceptibility of *S. maltophilia* to a wide range of antibiotics is due to the reduced permeability of the cell envelope as well as to the expression of several antibiotic resistance determinants encoded in its genome, including at least eight RND efflux pumps (15–17). The expression of each *S. maltophilia* RND efflux pump is regulated at different levels, which makes this organism a suitable model for the investigation of these systems. Among them, SmeYZ and SmeVWX are good examples of the transcriptional regulatory diversity present among *S. maltophilia* RND efflux pumps. The regulation of the expression of *smeYZ* is modulated by the two-component system (TCS) SmeRySy (18), while *smeVWX* expression is regulated by the LysR-type regulator SmeRv (19). In addition, whereas *smeYZ* is constitutively expressed at a significant level, hence contributing to the intrinsic resistance of *S. maltophilia* to aminoglycosides (20, 21), the level of *smeVWX* expression is not high enough to contribute to the intrinsic resistance of this microorganism (19) and SmeVWX-mediated antibiotic resistance is achieved only through its overexpression, as the consequence of mutations in its regulator, SmeRv (22).

As above stated, overexpression of MDR efflux pumps (and consequently antibiotic resistance) can be achieved through mutations in the regulatory elements controlling their expression. However, it is important to recall that there are occasions in which these antibiotic resistance determinants can be overexpressed without the need for a genetic change. Indeed, efflux pumps are expected to be expressed, when needed, in response to specific signals/cues (10). Consequently, there are situations and compounds capable of triggering the expression of these MDR systems, leading bacteria to display a phenotype of transient antibiotic resistance (23, 24). It is worth mentioning that besides their function as antibiotic resistance determinants, RND efflux pumps present a range of functions (11, 25–27) that include, among others, the bacterial response to host defenses (28–31), the modulation of the quorum-sensing (QS) signaling network (32–34), and the response to general stress situations (e.g., oxidative and nitrosative stress), acting as escape valves for the accumulation of toxic compounds or stress by-products (35).

RND efflux pumps are known to extrude a wide range of structurally different compounds, some of which are well characterized, especially in the case of clinically relevant antimicrobials (36). However, less is known about the efflux pump effectors. Given the above-mentioned functions of efflux pumps, most of the RND effectors already known have been discovered through the study of specific physiological processes wherein efflux pumps participate, such as the colonization of particular niches or the extrusion of a particular toxic compound (31, 37, 38). Expanding the knowledge of the set of RND inducer molecules could thus help in the characterization of MDR efflux systems, getting novel insights on their functional and ecological roles. Besides, the characterization of these effectors might also be useful for detecting possible situations of induced antibiotic resistance *in vivo*, which would not be detected by classical laboratory susceptibility tests.

Biolog phenotype microarrays consist of 96-well microplates developed for the determination of the bacterial response to a wide variety of chemical agents and nutrients, allowing testing nearly 2,000 phenotypes (39), which may help among several applications, including biofilm formation or the response to stressors, to decipher the phenotypes modulated by gene regulatory networks (40–48). In the current study, we have employed for the first time a different approach, based on the use of fluorescent reporters of the RND efflux pumps SmeVWX and SmeYZ, which present different levels of expression as well as different regulation patterns, to perform a wide screening of efflux pumps' inducer molecules, which could be applied as well to any other study on the regulation of gene expression. In addition, we have analyzed the effect of the induction of the expression of *smeVWX* and *smeYZ* on *S. maltophilia* susceptibility to the inducer compounds and to antibiotics currently used in clinical practice. Overall, our work provides new insights on the regulation of the expression and the role of the studied efflux pumps in the physiology of *S. maltophilia*.

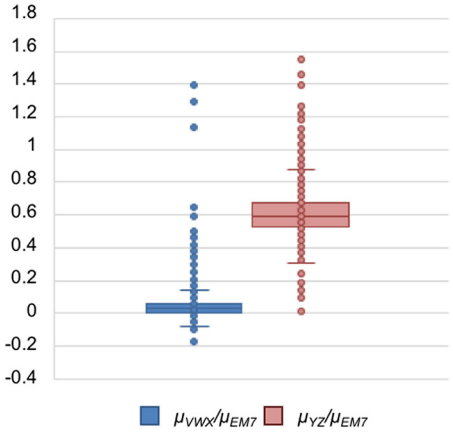


FIG 1 Normalized fluorescence production. Shown is a box plot representing the values of μ_{RND}/μ_{EM7} for all Biolog compounds and concentrations tested in plates PM11 to PM16 where there was observable growth. The upper whisker delimits the threshold above which outlier transcription activity is defined for *smeVWX* or *smeYZ* promoters and therefore efflux pump overexpression.

RESULTS

Characterization of reporter strains PBT03 (P_{EM7}) and PBT10 (P_{YZ}). In a previous study, we developed the reporter strain PBT02, which expresses a yellow fluorescent protein (YFP) under the control of the *smeVWX* promoter, in order to identify potential inducers of *smeVWX* expression (49). In the present work, two new YFP-based reporter strains have been developed as described in Materials and Methods: one containing the promoter of *smeYZ*, for measuring *smeYZ* expression (PBT10), and another which contains the constitutive promoter P_{EM7} (50) as a control for the normalization of the results (PBT03).

In order to test the proper functioning of the reporter strains, bacterial growth and YFP levels were determined. The fluorescence levels shown by the PBT10 strain were higher than those already described for the PBT02 strain (see Fig. S1 in the supplemental material), confirming that *smeYZ* is constitutively expressed in the wild-type *S. maltophilia* strain (20), while *smeVWX* expression was low, as reported previously (19). The YFP level shown by the PBT03 (P_{EM7}) strain was also assessed, and as expected, the expression was constitutive and suitable to be used as a positive control of fluorescence as well as for normalization purposes (Fig. S1).

Screening of inducers of MDR efflux pumps expression using Biolog phenotype plates. Biolog plates contain four different quantities (in different wells) of each tested compound. Nevertheless, information on these quantities is not available for users. The four wells for each of the tested compounds were named A, B, C, and D, with A being the well containing the highest amount of each compound. In the current work, plates PM11 to PM16 were chosen, making a total of 144 analyzed compounds (see Table S1). *S. maltophilia* reporter strains grew in at least one concentration for 142 of the tested compounds (Fig. S2). Those wells where *S. maltophilia* did not grow were discarded for further analysis.

The growth and the YFP fluorescence were recorded and analyzed in the presence of each of the tested compounds to obtain a proxy value of the transcriptional activity of the analyzed promoters. In the first step, the maximum specific rate of fluoresce production (μ) was determined as described in Materials and Methods for each promoter-compound-concentration combination (μ_{VWX}, μ_{YZ}, and μ_{EM7}). In the second step, the obtained values were normalized to those of the constitutive promoter and the values of normalized fluorescence production driven by each of the two analyzed promoters (μ_{VWX}/μ_{EM7} and μ_{YZ}/μ_{EM7}) were represented for every compound concentration in a box plot (Fig. 1). From these analyses, the upper whiskers of each distribution were used to establish the thresholds defining those situations where *smeVWX* and *smeYZ* were considered to be overexpressed. These thresholds were 0.14

TABLE 1 Normalized fluorescence production of P_{VWX} and P_{YZ} with Biolog compounds

Efflux pump, promoter analyzed	Plate	Compound	Fluorescence production of promoter normalized to that of μ_{EM7} in well[a]: A	B	C	D
SmeVWX, μ_{VWX}	11	Chlortetracycline		0.23	0.34	
		Cloxacillin				0.25
		Erythromycin				0.15
	12	D,L-Serine hydroxamate		0.17		
		Penimepicycline				0.28
		5-Fluoroorotic acid				0.21
		Dodecyltrimethylammonium bromide				0.17
		Spiramycin				0.16
		Vancomycin				0.15
	13	Thallium (I) acetate	0.17			
		Manganese chloride		0.19		0.65
		Potassium chromate				0.42
		Tylosin				0.30
	14	Iodoacetate		0.50	0.22	
	15	Menadione		0.38	0.59	
		5,7-Dichloro-8-hydroxyquinaldine			0.27	0.51
		Methyl viologen				0.18
		Nordihydroguaiaretic acid				0.18
	16	Sodium selenite	1.14	1.30	1.40	1.40
		5-Chloro-7-iodo-8-hydroxyquinoline	0.22	0.46	0.45	0.38
		Cinoxacin	0.19			
		Cetylpyridinium chloride	0.16			
		Protamine sulfate				0.18
SmeYz, μ_{YZ}						
	11	Chloramphenicol		1.06	0.99	
		Erythromycin		0.96	1.22	1.27
		Lincomycin		0.98	1.30	1.49
	12	5-Fluoroorotic acid	1.18	1.08	0.95	0.98
		D,L-Serine hydroxamate			1.02	
		Penimepicycline				1.09
		Spiramycin		0.93	0.97	1.42
		Sulfadiazine				0.98
		Sulfathiazole				1.12
	13	Azlocillin				0.90
		Dequalinium chloride				0.92
		Rolitetracycline				1.03
		Tylosin			1.40	1.58
		Boric acid		0.96		
	14	Chloramphenicol			1.13	1.06
		Sodium metaborate	0.99			
		Fusidic acid	1.12	0.92		
	15	Oleandomycin		1.11	1.04	
		Puromycin				0.91
	16	Cetylpyridinium chloride	0.98			
		Cinoxacin	1.46			
		Protamine sulfate	1.28	1.41	1.47	1.55

[a]A, B, C, and D refer to the four wells per compound, with A being the well with the highest concentration and D the well with the lowest concentration of each compound.

for μ_{VWX}/μ_{EM7} and 0.89 for μ_{YZ}/μ_{EM7}, respectively. All the compounds and concentrations that led to the overexpression of *smeVWX* or *smeYZ* following these rules are listed in Table 1. In the case of the SmeVWX efflux pump, the value of μ_{VWX}/μ_{EM7} for 23 compounds and 34 concentrations was found to exceed the threshold, while the value of μ_{YZ}/μ_{EM7} was above the threshold in the case of the SmeYZ efflux pump for 22 compounds and 39 concentrations.

Confirmation of the increased expression of *smeVWX* and *smeYZ* in the presence of potential inducers. Among those Biolog compounds that increased the YFP levels for each of the studied efflux pumps, some of those for which the induction was higher in order to validate the results were selected. As possible inducers of *smeVWX*, iodoacetate, clioquinol (5-chloro-7-iodo-8-hydroxyquinoline), and sodium selenite were

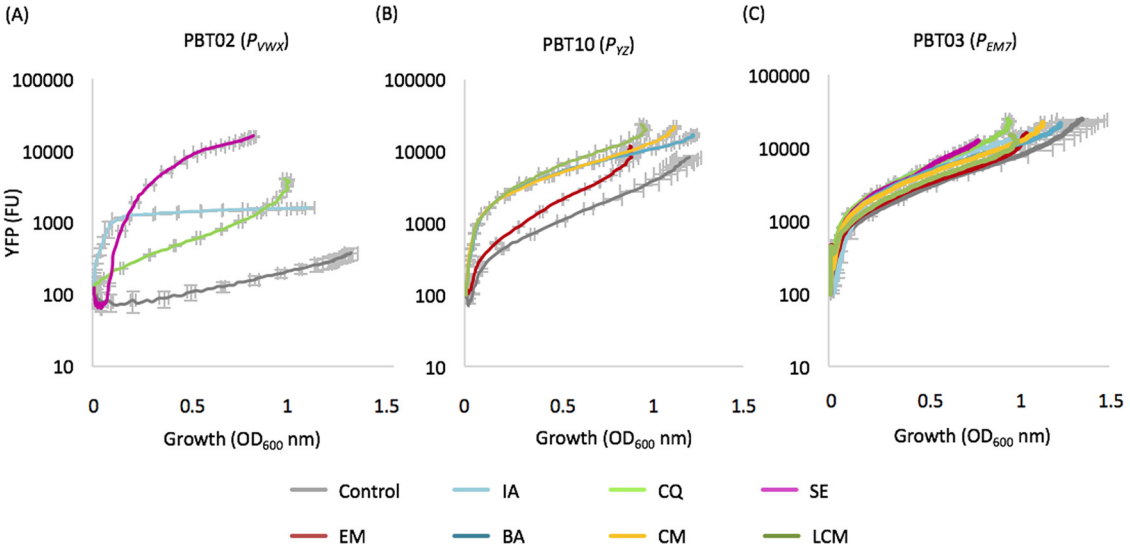
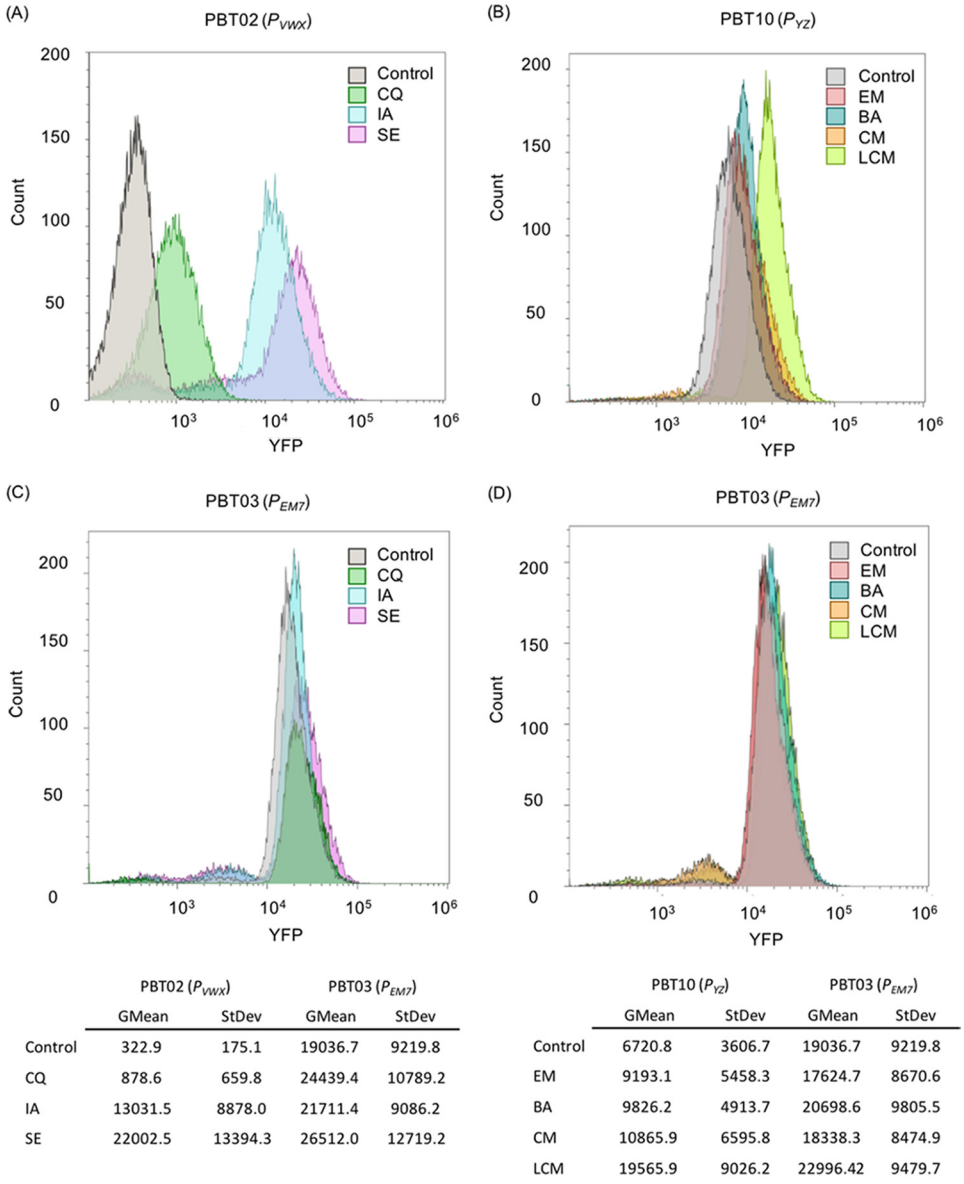


FIG 2 Growth and fluorescence values produced by the reporter strains PBT02 (P_{VWX}), PBT10 (P_{YZ}), and PBT03 (P_{EM7}) after incubation with the selected putative inducers. (A) Expression of *smeVWX* during 20 h of incubation in the presence of iodoacetate (0.5 mM), clioquinol (78 μM), or sodium selenite (0.5 mM). (B) Expression of *smeYZ* during 20 h of incubation in the presence of erythromycin (8 μg/ml), boric acid (6.25 mM), chloramphenicol (1 μg/ml), or lincomycin (128 μg/ml). (C) P_{EM7} expression during 20 h of incubation with all the compounds at the above-mentioned concentrations. Error bars show SDs from three independent replicates. FU, fluorescence units; CQ, clioquinol; IA, iodoacetate; SE, sodium selenite; BA, boric acid; CM, chloramphenicol; EM, erythromycin; LCM, lincomycin.

selected; boric acid, chloramphenicol, erythromycin, and lincomycin were chosen as potential *smeYZ* inducers. Since the quantity of the inducing compounds present in the Biolog plate was unknown, we first determined the MICs of the potential inducers in order to establish the right concentrations to be used in the next studies. These concentrations were as follows: iodoacetate, 1 mM; clioquinol, 0.065 mM; sodium selenite, ≥200 mM; boric acid, 25 mM; erythromycin, 256 μg/ml; chloramphenicol, 4 μg/ml; and lincomycin, 4,096 μg/ml. Three concentrations below the MIC value were selected to further study the role of these compounds as inducers of the expression of the tested efflux pumps.

To carry out the experiment, the reporter strains PBT02 (P_{VWX}) and PBT10 (P_{YZ}) were incubated with their respective putative inducers for 20 h. The PBT03 (P_{EM7}) strain was also incubated with all the compounds and results were used as fluorescence controls. Figure 2 shows the YFP values and growth obtained for the PBT02 and PBT10 strains, using the concentration for which the highest induction was obtained and bacterial growth was less compromised in each case. These concentrations were 0.5 mM sodium selenite, 78 μM clioquinol, 0.5 mM iodoacetate, 6.25 mM boric acid, 8 μg/ml of erythromycin, 1 μg/ml of chloramphenicol, and 128 μg/ml of lincomycin. In agreement with the data obtained using the Biolog plates, iodoacetate, clioquinol, and sodium selenite increased YFP production through the induction of the *smeVWX* promoter in the PBT02 strain, in comparison with the fluorescence observed in control medium without inducer (Fig. 2A). Meanwhile, erythromycin, boric acid, chloramphenicol, and lincomycin increased the production of YFP through the induction of the *smeYZ* promoter in the PBT10 strain, compared with the strain grown in the control medium (Fig. 2B). PBT03 did not show any difference in YFP production when incubated with and without the different compounds (Fig. 2C). These results confirm that the observed fluorescence increase observed in the Biolog plates for both reporter strains PBT02 and PBT10, when grown in the presence of the above-mentioned potential effectors, is due to the induction of the promoters of the tested efflux pumps.

Single-cell analysis of the induction of the expression of MDR efflux pumps. In order to examine the induction of *smeVWX* and *smeYZ* expression at the single-cell level, we measured by flow cytometry the YFP values of the two reporter strains PBT02



	PBT02 (P_{VWX})		PBT03 (P_{EM7})	
	GMean	StDev	GMean	StDev
Control	322.9	175.1	19036.7	9219.8
CQ	878.6	659.8	24439.4	10789.2
IA	13031.5	8878.0	21711.4	9086.2
SE	22002.5	13394.3	26512.0	12719.2

	PBT10 (P_{YZ})		PBT03 (P_{EM7})	
	GMean	StDev	GMean	StDev
Control	6720.8	3606.7	19036.7	9219.8
EM	9193.1	5458.3	17624.7	8670.6
BA	9826.2	4913.7	20698.6	9805.5
CM	10865.9	6595.8	18338.3	8474.9
LCM	19565.9	9026.2	22996.42	9479.7

FIG 3 Population analysis of *smeVWX* and *smeYZ* expression in the presence of the selected putative inducers. YFP production was analyzed by flow cytometry. (A) Expression profile of the *smeVWX* promoter in strain PBT02 treated with clioquinol (78 μM), iodoacetate (0.5 mM), or sodium selenite (0.5 mM). (B) Expression profile of the *smeYZ* promoter in strain PBT10 treated with erythromycin (8 μg/ml), boric acid (6.25 mM), chloramphenicol (1 μg/ml), or lincomycin (128 μg/ml). (C and D) Expression profile of the P_{EM7} promoter in strain PBT03 treated with all the different compounds at the above-mentioned concentrations. Gray populations represent in all cases the basal promoters' expressions in the absence of any compound. Geometric mean (GMean) and standard deviation (StDev) were calculated for each population using Kaluza 1.5 software.

and PBT10 after 90 min of incubation with their respective inducers when cells reached exponential growth phase (optical density at 600 nm [OD_{600}] $\approx$ 0.6) (Fig. 3). Measuring induction at exponential growth phase lessens potential interferences with the fluorescence signal, growth phase, or compound degradation that could happen over time. PBT03 was also treated with the entire set of compounds as a control (Fig. 3C and D). As shown in Fig. 3A, the expression of *smeVWX* in the PBT02 population was higher in the presence of clioquinol, iodoacetate, or sodium selenite than in the untreated population. The same results were obtained when the PBT10 strain was incubated with boric acid, erythromycin, chloramphenicol, or lincomycin, showing that *smeYZ* expression is increased in the presence of these compounds (Fig. 3B). For all the compounds,

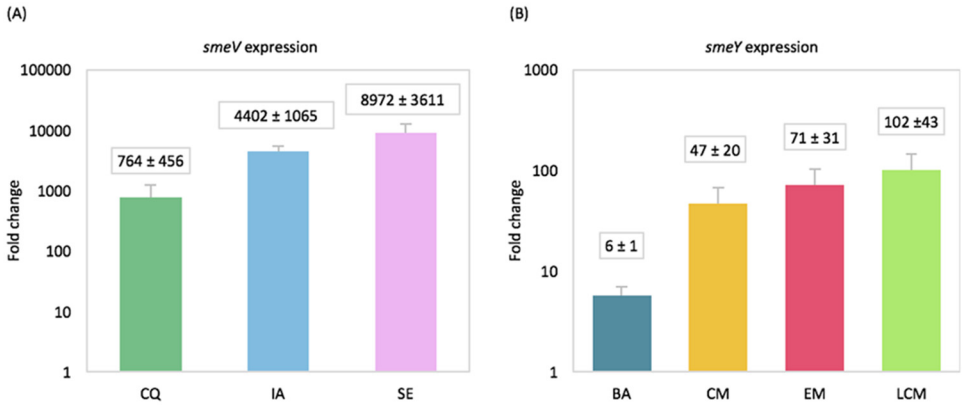


FIG 4 Effect of putative inducers on the mRNA levels of *smeV* and *smeY*. Expresion of *smeV* and *smeY* was measured in the presence and in the absence of their potential inducers by real-time RT-PCR. (A) *smeV* expression in strain D457 after 90 min of incubation with clioquinol (78 μM), iodoacetate (0.5 mM), or sodium selenite (0.5 mM). (B) *smeY* expression in D457 after 90 min of incubation with boric acid (6.25 mM), chloramphenicol (1 μg/ml), erythromycin (8 μg/ml), or lincomycin (128 μg/ml). Fold changes were estimated with respect to the values obtained for the untreated D457 strain. Error bars show SDs derived from three independent experiments.

a unimodal distribution of the level of YFP was seen in the population, indicating that all cells presented an increased expression of the corresponding efflux pump in the presence of the inducers. In addition, the averages of fluorescence corresponded with those obtained when induction was measured along the growing cycle (see above), showing that induction of the analyzed efflux pumps is produced at mid-exponential growth phase.

Analysis of the expression of efflux pumps in the presence of inducers at the mRNA level. For further confirmation of the results, the mRNA levels of *smeV* and *smeY* were quantified by real-time reverse transcription-PCR (RT-PCR) in the wild-type strain *S. maltophilia* D457 in the presence of the different inducer compounds. As shown in Fig. 4A, *smeV* expression increased 764-, 4,402-, and 8,972-fold in the presence of clioquinol, iodoacetate, and sodium selenite, respectively. The same effect can be observed in Fig. 4B, where *smeY* expression increased 6-, 47-, 71-, and 102-fold in the presence of boric acid, chloramphenicol, erythromycin, and lincomycin, respectively. These data confirm that the compounds selected from the analysis using Biolog plates, namely, clioquinol, iodoacetate, and sodium selenite, are *smeVWX* inducers, and boric acid and the antibiotics chloramphenicol, erythromycin, and lincomycin are able to trigger *smeYZ* expression.

Deletion of either *smeW* or *smeZ* does not alter the susceptibility of *S. maltophilia* to their corresponding inducers. Since the *smeVWX* and *smeYZ* inducers described here are antimicrobials, we wanted to elucidate whether these compounds were also substrates of their respective efflux pumps. To test this, the growth of D457 and MBS704 (Δ*smeW*) in the presence of clioquinol (78 μM), iodoacetate (0.5 mM), or sodium selenite (0.5 mM) was recorded. The same experiment was performed for *smeYZ* inducers, growing D457 and PBT100 (Δ*smeZ*) strains in the presence of boric acid (6.25 mM), chloramphenicol (1 μg/ml), erythromycin (8 μg/ml), or lincomycin (128 μg/ml). If these compounds are extruded through the efflux pumps, it is expected that both *smeW*- and *smeZ*-defective mutants should have an increased deficiency in their growth in the presence of inducers compared with the wild-type strain. As shown in Fig. 5, no relevant growth differences were observed between the wild-type and the efflux pump-defective strains in the presence of the different inducer compounds, despite the fact that all the analyzed compounds impaired bacterial growth. It is generally assumed that efflux pump inducer molecules are also extruded by the efflux system; the efflux pump hence confers resistance to such effectors if they are toxic (51–54). However, the data obtained in this work suggest that efflux pumps do not always confer resistance

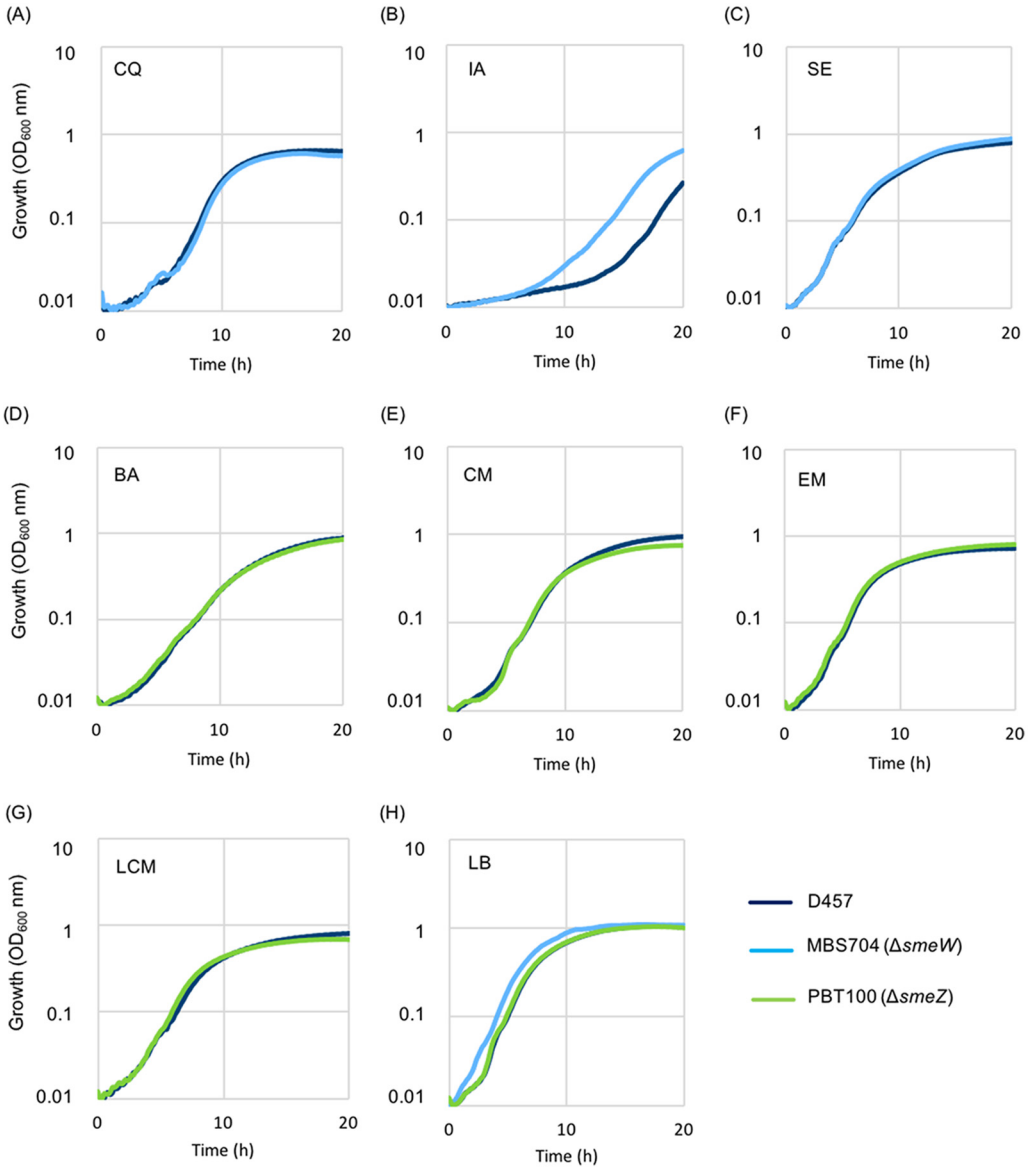


FIG 5 Effects of *smeW* and *smeZ* on the susceptibility of *S. maltophilia* to the inducers of these efflux pumps. The strains D457, MBS704 (Δ*smeW*), and PBT100 (Δ*smeZ*) were grown in LB medium as a control (H). D457 and MBS704 were grown in the presence of clioquinol (78 μM) (A), iodoacetate (0.5 mM) (B), and selenite (0.5 mM) (C). D457 and PBT100 were grown in the presence of boric acid (6.25 mM) (D), chloramphenicol (1 μg/ml) (E), erythromycin (8 μg/ml) (F), and lincomycin (128 μg/ml) (G). Represented values correspond to the means calculated from three independent replicates.

to their inducers, likely because in this case, these compounds are not substrates of the efflux systems they induce.

The overexpression of MDR efflux pumps promotes transient resistance to antibiotics. It is known that overexpression of *smeVWX* contributes to the acquired resistance to chloramphenicol and quinolones of *S. maltophilia* (19), while SmeYZ is able to extrude aminoglycosides, contributing to intrinsic resistance to them (20, 21). Since new inducer compounds have been identified for both RND efflux pumps, we wondered whether the susceptibility of *S. maltophilia* to known antibiotic substrates of these MDR determinants would be altered in the presence of these putative effectors.

In order to test the contribution of the SmeVWX efflux pump to transient resistance, bacterial growth was measured in the presence and in the absence of ofloxacin, and with or without the inducer sodium selenite, using both D457 and MBS704 (Δ*smeW*) strains. As shown in Fig. 6A and B, sodium selenite, at the tested concentration, did not

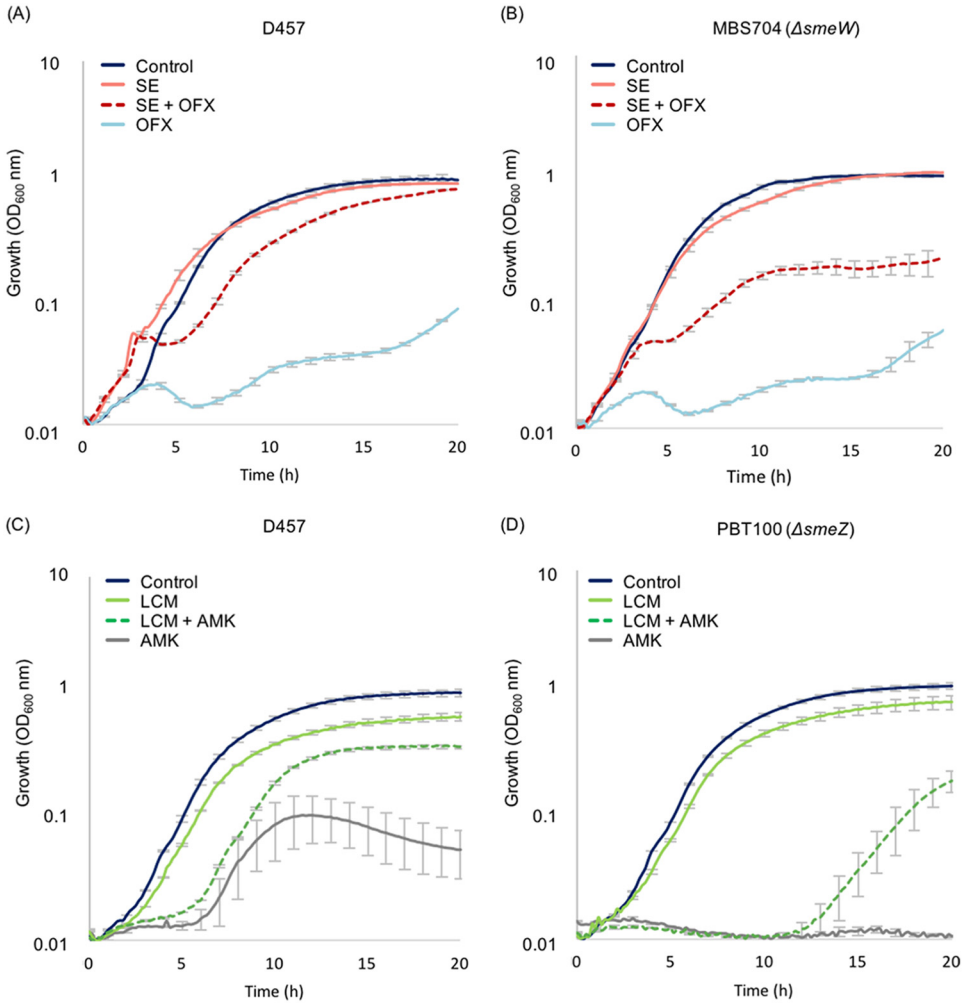


FIG 6 Effects of the inducer compounds sodium selenite and lincomycin in the transient resistance of *S. maltophilia* to antibiotics. Growth curves were performed in the presence of ofloxacin (OFX), sodium selenite (SE), and both ofloxacin and sodium selenite for strains D457 (A) and MBS704 (Δ*smeW*) (B). Growth curves were also performed in the presence of amikacin (AMK), lincomycin (LCM), and both amikacin and lincomycin in strains D457 (C) and PBT100 (Δ*smeZ*) (D). Growth in LB medium was used as a control. Error bars show SDs derived from three independent replicates.

compromise the growth of either strain significantly, whereas ofloxacin impaired the growth of both D457 and MBS704. However, the combination of ofloxacin together with sodium selenite did not impede growth of D457. With the aim of assessing whether SmeYZ may contribute to transient resistance during growth in the presence of its potential effectors, a similar experiment was performed in which the D457 and PBT100 (Δ*smeZ*) strains were grown in the presence or absence of the efflux pump substrate amikacin, with or without the inducer lincomycin. As shown in Fig. 6C and D, lincomycin slightly reduced the growth of both strains due to its toxic effect. At the tested concentration of amikacin, the growth was compromised for both strains; however, the presence of lincomycin diminished the inhibitory effect of amikacin. These data suggest that both MDR pumps are involved in transient resistance to antibiotics when inducers are present, since sodium selenite and lincomycin are able to induce *smeVWX* and *smeYZ*, respectively, changing the susceptibility of D457 to their antibiotic substrates.

DISCUSSION

The conditions that lead to the induction of the expression of MDR efflux pumps, which consequently could give rise to transient antibiotic resistance, have not been

fully explored, and their detection through the use of classical susceptibility methods is usually difficult (6, 23).

In a previous screening using the PBT02 (P_{vwx}) strain, we determined that vitamin K_3, among 30 tested compounds, was an inducer of the SmeVWX system (49). In the current study, around 40 compounds were selected as potential candidates for being *smeVWX* or *smeYZ* inducers after screening of 144 compounds employing Biolog phenotype microarrays. Among all the potential inducer compounds derived from the Biolog data analysis, clioquinol (5-chloro-7-iodo-8-hydroxyquinoline), iodoacetate, and sodium selenite (*smeVWX* inducer candidates) and boric acid, chloramphenicol, erythromycin, and lincomycin (*smeYZ* inducer candidates) were selected for further analysis. The fluorescence data obtained through the use of Biolog plates were confirmed by testing cognate concentrations of the selected agents, as well as by flow cytometry at mid-exponential growth phase and by RT-PCR after exposure to the corresponding compounds, obtaining similar results for the expression level. Hence, the Biolog technology linked to the use of florescence reporters has allowed us to successfully identify a set of inducer molecules of the expression of RND efflux pumps.

Although the main purpose of the current work was to identify RND efflux pumps' inducers, the combination of Biolog microarrays and fluorescent reporters could also be employed for the search of inhibitors of the expression of these efflux systems, which would help to reduce the emergence and spread of antibiotic resistance. Moreover, our approach could be broadly used in the screening of effector molecules for any transcriptional regulatory system (55).

Besides contributing to antibiotic resistance, MDR efflux pumps are involved in different aspects of bacterial physiology, including bacterial interaction with hosts (human, animals, and plants) or detoxification of cellular toxic metabolites (26). MDR efflux pumps can also take part in general mechanisms of response to cellular stress that contribute to ameliorate the adverse effects caused by stressor agents or stress by-products (e.g., oxidative stress or envelope stress) (35, 56). We hypothesize that when MDR efflux systems participate in this global cellular stress response, a general common inducer mechanism of stress could be identified through the analysis of their inducer compounds, despite their structural diversity. As described here, clioquinol, iodoacetate, and sodium selenite have been identified as *smeVWX* inducers, in addition to the previously described vitamin K_3 (49). Although these compounds generate oxidative stress (57–60), except iodoacetate (61), we determined in our previous work that *tert*-butyl hydroperoxide, another oxidative stress agent, was not able to induce *smeVWX* expression (49). However, all of the identified inducer compounds are associated with thiol reactivity. Iodoacetate is an alkylating reagent that modifies thiol groups in proteins by S-carboxymethylation (61); selenite is known to catalyze the oxidation of thiol groups and to induce protein aggregation (62); clioquinol, as a Cu ionophore, can deliver metal ions into cells, where it exerts its activity through the interaction with thiol and amino groups (63); and, finally, vitamin K_3 (menadione) contributes to redox cycling and has alkylating properties, reacting as well with thiol groups (60). All of this evidence suggests that the *smeVWX* induction mechanism might be related, at least in part, to the thiol reactivity of the inducer compounds.

In the case of the SmeYZ efflux pump, boric acid, erythromycin, chloramphenicol, and lincomycin were identified as inducers. Erythromycin targets the 50S subunit of the bacterial ribosome and inhibits the nascent chain elongation (64). Chloramphenicol targets the 50S subunit of the ribosome by its binding to the peptidyl transferase center (PTC), where peptide bond formations happen (65). Both erythromycin and chloramphenicol are also known to directly inhibit the biogenesis of the ribosomal 50S subunit (66). The third identified antibiotic inducer, lincomycin, also targets the 50S subunit of the ribosome by inhibiting peptide bond formation (67). *smeYZ* was also found to be induced by boric acid, which is not an antibiotic but impairs the acylation of tRNAs and inhibits protein synthesis (68). All of these compounds share a mechanism of action, suggesting that SmeYZ mechanism of induction might be related to protein synthesis inhibition. Supporting this possibility, other compounds present in the Biolog plates,

such as oleandomycin, spiramycin, tylosin, penimepiclyne, and fusidic acid, whose mechanism of action is also the inhibition of protein synthesis, were found to increase the YFP levels produced by the PBT10 strain (Table 1). These data reinforce the hypothesis that ribosome-stalling stress could be a signal that triggers *smeYZ* expression, as happens with other RND efflux pumps, such as MexXY of *Pseudomonas aeruginosa* (69).

It has been described that RND efflux pumps extrude several of their known inducers, such as aminoglycosides in the case of MexXY-OprM in *P. aeruginosa* (70), or triclosan in the case of SmeDEF in *S. maltophilia* (51), a protection mechanism against these toxic agents. However, in this work, we have shown that the lack of either SmeVWX or SmeYZ does not affect the growth of *S. maltophilia* in the presence of their toxic inducers (Fig. 5). Different circumstances might explain this apparent contradiction; one possibility is that the concentration for induction is lower than or similar to the toxic concentration of the tested compound, not allowing detection of an effect (45). Also, the bacterium can exhibit more efficient mechanisms, (e.g., other efflux pumps) able to extrude or detoxify the same compounds, in which case these mechanisms must be removed for analyzing the less proficient ones (71). In addition, the possibility that an effector is not extruded by the efflux pump it induces cannot be disregarded. In this respect, it is worth noticing that the substrates and the inducers of a given efflux pump are frequently structurally diverse (26), although they can interfere with the same cell machinery or target. Indeed, we propose two common mechanisms of stress generated by a set of RND inducers, each one inducing the expression of an efflux pump. It is important to consider that RND efflux pumps mainly extrude substrates that are located in the periplasm or in the bacterial inner membrane (72), while the regulation of these systems takes place in the cytoplasm (73). This differentiated compartmentalization may justify the possibility that RND inducers might not always be substrates of efflux pumps. This means that in some situations, MDR efflux pumps might be overexpressed despite the fact that there is no apparent advantage for bacteria. However, the presence of the inducer may imply a situation of transient resistance to other toxic compounds, which represents a benefit under some conditions. We have assessed this situation for clinically useful antibiotics that are RND efflux pumps substrates using sodium selenite and lincomycin, the strongest inducers of SmeVWX and SmeYZ efflux pumps, respectively. Both inducer molecules were able to promote transient reduced susceptibility to ofloxacin (SmeVWX substrate) or amikacin (SmeYZ substrate).

This is a fact to take into account regarding clinical situations, since some of these inducer molecules could be provided during treatment of *S. maltophilia* infection. For instance, sodium selenite has been recently administered during a phase I clinical trial in terminal cancer patients (74) due to its cytotoxic effect on proliferating cancer cells. Since these patients are very vulnerable to infections caused by MDR Gram-negative bacteria, such as *S. maltophilia* (75), the use of sodium selenite could lead to the overexpression of *smeVWX* and thus transient resistance to its substrates. The clinical consequences of this situation need to be further investigated.

Through the combination of Biolog phenotype microarrays and fluorescence-based reporter strains, we have developed a new high-throughput methodology to identify MDR efflux pump inducer compounds. The common mechanism of action of the detected inducer molecules has allowed us to establish possible mechanisms of induction of both *smeVWX* and *smeYZ* in *S. maltophilia*. *smeVWX* is likely induced by compounds related to thiol reactivity, while *smeYZ* is induced by agents that target the ribosome, suggesting a relationship between the expression of the efflux pump and the inhibition of protein synthesis. Together with already published works (76, 77), these results indicate that MDR efflux pump expression is triggered not always by a specific compound but by stress signals generated by certain molecules with similar mechanisms of action.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All plasmids and strains derived from this study are listed in Table 2. All experiments were performed using LB medium at 37°C. The following antibiotics were added when required: streptomycin (50 μg/ml) for *Escherichia coli* harboring the pSEVA4413 plasmid, ampicillin (100 μg/ml) for *E. coli* containing the pGEM-T Easy vector and the pGEM-T-derived plasmids pPBT02 and pPBT11, and kanamycin (50 μg/ml and 500 μg/ml) for *E. coli* and *S. maltophilia*, respectively, for the selection of pSEVA237Y and the derived plasmids pPBT04, pPBT05, and pPBT08. Medium was supplemented with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 80 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) for the induction and detection of β-galactosidase production.

Plasmid constructions. The PBT10 strain, which harbors the reporter plasmid pPBT08 containing the *smeYZ* promoter region, was obtained as described previously (49) using the primers SmeYZ_F (5′-GAATTCGGCGGGGCCGTAAG-3′; EcoRI site underlined) and SmeYZ_R (5′-AAGCTTTGCTGTGCACAATG-3′; HindIII site underlined). In order to obtain the PBT03 strain, which harbors the pPBT05 plasmid with the constitutive promoter P_{EM7}, the pSEVA4413 plasmid was digested with PacI and HindIII (New England BioLabs). The product corresponding to the P_{EM7} promoter was purified from a 1% agarose gel using a DNA purification kit (GE Healthcare) and cloned into the pSEVA237Y plasmid, previously digested with the same restriction enzymes. The resulting plasmid, pPBT05, was introduced by transformation in *E. coli* CC118λ*pir*. Then pPBT05 was introduced into *S. maltophilia* D457 by tripartite mating as previously described (49).

Biolog phenotype microarray assay. The 96-well plates PM11 to PM16 from the Biolog phenotypic microarray (PM) system (Biolog), which contain a variety of chemical agents (listed in Table S1), including antimicrobials (78), were used for the screening. To carry out the experiment, 100 μl of LB medium was added to each well and plates were incubated at room temperature under constant shaking for 2 h in order to dissolve the different dried compounds in the medium before inoculating the bacterial cells. Ten microliters of an overnight cell culture was added to each well to a final OD_{600} of 0.01. Plates were then incubated at 37°C for 20 h in a Tecan Spark 10M plate reader (Tecan). Growth (OD_{600}) and fluorescence (excitation wavelength at 508 nm and emission wavelength at 540 nm) were measured every 10 min. Plates were shaken for 5 s before each measurement.

Normalization of the results. The obtained time courses of fluorescence determinations (see above) were fitted to a first-order differential equation, analogously to the one that represents bacterial growth, $F(t) = F_0\, e^{\mu\, t}$ [where $F(t)$ is fluorescence across the time, F_0 is initial fluorescence, μ is maximum specific rate of fluorescence production (per hour), and t is time (in hours)]. Using this equation, μ was obtained for every promoter-compound-concentration combination, giving rise to a set of μ_{YZ}, μ_{VWX}, and μ_{EM7} values. Afterwards, the normalized values of fluorescence production (μ_{VWX}/μ_{EM7} and μ_{YZ}/μ_{EM7}) were obtained, dividing μ_{YZ} and μ_{VWX} by μ_{EM7} for each compound and concentration. This step is required to avoid any effect of the toxic compound on the fluorescence signal, which may be caused by itself or by its action on the growth rate, being inversely related to the fluorescence signal (79). To set a threshold of the normalized values of fluorescence production, which could indicate that the expression is increased (overexpression), a box plot of every normalized value of fluorescence production for all the compounds and concentrations was obtained. Then the threshold to define outlier values, and therefore values which could indicate overexpression, was set according to the formula $Q_3 + 1.5 \times \mathrm{IQR}$, where Q_3 is the upper quartile and IQR the interquartile range of each data set. These values were 0.14 for μ_{VWX}/μ_{EM7} and 0.89 for μ_{YZ}/μ_{EM}.

Confirmation of the induction of gene expression. Sodium selenite, clioquinol (5-chloro-7-iodo-8-hydroxyquinoline), and iodoacetate were selected as possible inducers of the SmeVWX efflux pump, while boric acid and the antibiotics erythromycin, chloramphenicol, and lincomycin were chosen as possible inducers of the SmeYZ efflux pump. First, the MIC was determined for each compound by microdilution using 96-well plates (Nunclon Delta Surface). Ten microliters of each overnight cell culture were added to 140 μl of medium containing different concentrations of the selected compound to a final OD_{600} of 0.01. Plates were incubated at 37°C for 20 h without shaking. The MIC value was defined as the lowest concentration at which bacterial growth was not observed in the presence of the tested compound.

Three concentrations of each compound were chosen for the induction assay considering the obtained MIC values, as follows: sodium selenite, 0.25, 0.5, and 1 mM; clioquinol, 78, 156, and 312 μM; iodoacetate, 0.25, 0.5, and 1 mM; boric acid, 3.125, 6.25, and 12.5 mM; erythromycin, 4, 8, and 16 μg/ml; chloramphenicol, 0.5, 1, and 2 μg/ml; and lincomycin, 64, 128, and 256 μg/ml. Ten microliters of each bacterial culture was added to 140 μl of medium to a final OD_{600} of 0.01 using Corning Costar 96-well black clear-bottom plates (Corning Incorporated). Plates were incubated at 37°C for 20 h, and growth (OD_{600}) and fluorescence were monitored every 10 min in the Tecan Spark 10M plate reader (Tecan). Plates were shaken for 5 s every 10 min before each measurement. An excitation wavelength at 508 nm and emission wavelength at 540 nm were set for YFP detection.

Flow cytometry assays. *S. maltophilia* reporter strains PBT02 (P_{VWX}), PBT03 (P_{EM7}), and PBT10 (P_{YZ}) were inoculated in 100-ml Erlenmeyer flasks with 20 ml of LB medium to a final OD_{600} of 0.01 and incubated at 37°C with shaking. When bacterial cultures reached mid-exponential growth phase ($OD_{600} \approx 0.6$), induction with the different compounds was tested using the following concentrations: 0.5 mM sodium selenite, 78 μM clioquinol, 0.5 mM iodoacetate, 6.25 mM boric acid, 8 μg/ml of erythromycin, 1 μg/ml of chloramphenicol, and 128 μg/ml of lincomycin. A bacterial culture with no compound was used as a control. Cells were then incubated with shaking for 90 min at 37°C. One milliliter of each culture was centrifuged at 13,000 rpm for 1 min at room temperature. Cells were washed with 500 μl of

TABLE 2 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Bacterial strains		
E. coli		
OmniMAX	Strain used in transformation. F′ *mcrA* Δ(*mrr-hsd*RMS-*mcrBC*) Φ80(*lacZ*)ΔM15 Δ(*lacZ*YA-*argF*) *U169 endA1 supE44 thi-1 gyrA96 relA1 deoR tonA panD*	Invitrogen, Life Technologies
CC118λ*pir*	Donor cell in conjugation. Strain CC118 lysogenized with λ*pir* phage (Tc) Δ(*ara-leu*) *araD* Δ*lac*X74 *galE galK phoA20 thi-1 rpsE rpoB argE* (Am) *recA1*	82
1047(pRK2013)	Helper cell in conjugation harboring pRK2013 (Kan^r) plasmid	83
S. maltophilia		
D457	Clinical strain	84
PBT02	D457 harboring the pPBT04 (P_{VWX}) plasmid	49
PBT03	D457 harboring the pPBT05 (P_{EM7}) plasmid	This work
PBT10	D457 harboring the pPBT08 (P_{YZ}) plasmid	This work
MBS704	D457 Δ*smeW*	49
PBT100	D457 Δ*smeZ*	This work
Plasmids		
pGEM-T Easy vector	Cloning vector; Amp^r	Promega
pSEVA237Y	Plasmid containing YFP protein; replication origin pBBR1; Kan^r	85
pSEVA4413	Plasmid containing P_{EM7} promoter; replication origin pRO1600/ColE1; Sm^r	85
pPBT02	pGEMT-derived plasmid containing *smeVWX* promoter region	49
pPBT11	pGEMT-derived plasmid containing the *smeYZ* promoter region	This work
pPBT04	pSEVA237Y-derived plasmid containing the *smeVWX* promoter region	49
pPBT05	pSEVA237Y-derived plasmid containing the P_{EM7} promoter	This work
pPBT08	pSEVA237Y-derived plasmid containing the *smeYZ* promoter region	This work
pEX18Tc	Gene replacement vector; *sacB* Tet^r	81
pZAB7	pEX18Tc-derived plasmid containing the 5′ and 3′ regions of the *smeZ* gene	This work

phosphate-buffered saline (PBS) and centrifuged as indicated above. The bacterial pellets were suspended with 300 μl of 0.4% paraformaldehyde and incubated at room temperature for 10 min. Cells were then centrifuged as indicated above and suspended in 1 ml of PBS. In order to avoid false-positive signals, all the media and buffers employed were filtered through 0.22-μm-pore filters (Corning Incorporated). With the aim of measuring YFP production at the single-cell level, samples of each reporter strain containing 20,000 cells were analyzed using a Gallios flow cytometer (Beckman Coulter). Data processing was accomplished with Kaluza 1.5 software (Beckman Coulter).

RNA preparation and real-time RT-PCR. RNA was obtained as described previously, with some modifications (49). Briefly, a 100-ml Erlenmeyer flask with 20 ml of LB medium was inoculated with an overnight culture of *S. maltophilia* D457 to reach a final OD_{600} of 0.01. Cell cultures were incubated at 37°C in agitation until they reached mid-exponential growth phase ($OD_{600} \approx 0.6$). At this point, the induction assay was performed, adding the different compounds at the concentrations required: 0.5 mM sodium selenite, 78 μM clioquinol, 0.5 mM iodoacetate, 6.25 mM boric acid, 8 μg/ml of erythromycin, 1 μg/ml of chloramphenicol, and 128 μg/ml of lincomycin. A bacterial culture with no compound was used as a control. Cultures were incubated with shaking for 90 min. Ten milliliters of each culture was taken and centrifuged at 8,000 rpm for 20 min at 4°C. RNA extraction was performed as described previously (49), and cDNA was obtained using a high-capacity cDNA reverse transcription kit (Applied Biosystems). One hundred nanograms of cDNA was used for each reaction. Real-time RT-PCR was performed in an ABI Prism 7500 real-time system (Applied Biosystem) using the Power SYBR green PCR master mix (Applied Biosystem). Primers RT-SmeV.L (5′-GTCGACTTCCTCGACAACC-3′) and RT-SmeV.R (5′-TTGCCATCCTTGTCTACCAC-3′) were used to amplify *smeV*, primers RT-SmeY.L (5′-CATTGGTGACCGAAGGTG-3′) and RT-SmeY.R (5′-TTGATACCGGAGAACAGCAG-3′) were used to amplify *smeY*, and primers RT-ftsZ.L (5′-ATGGTCAACTCGGCAGTG-3′) and RT-ftsZ.R (5′-CGGTGATGAACACCATGTC-3′) were used to amplify the housekeeping gene *ftsZ*. Relative changes in gene expression were determined according to the threshold cycle ($2^{-\Delta\Delta CT}$) method (80). Mean values were obtained from three independent replicates in each experiment.

Deletion of *smeZ*. A partial deletion of the *smeZ* gene was performed in *S. maltophilia* D457 through homologous recombination. To that end, 545-bp (ZA) and 549-bp (ZB) fragments from the 5′ end and 3′ end of the *smeZ* gene, respectively, were amplified by PCR using primers ZAF (5′-GAATTCATGGCACGTTTCTTCATCGATCGCCCGGTGTTCGC-3′; EcoRI site underlined) and ZAR (5′-ATCGACAACAACAGCAGCCATGCTCGGCACCGAACAACTG-3′) and primers ZBF (5′-CAGTTGTTCGGTGCCGAGCATGGCTGCTGTTGTTGTCGAT-3′) and ZBR (5′-GAATTCTCAACGATGTTCCGTTCCATCCACGGTTCCTCCCGGC-3′; EcoRI site underlined). An overlapping PCR was performed with ZA and ZB fragments as the template using ZAF and ZBR primers, yielding a 1,000-bp fragment (ZAB). The ZAB fragment was purified from a 1% agarose gel using a DNA purification kit (GE Healthcare) and cloned into pGEM-T Easy (Promega) following the manufacturer's protocol. The sequence was confirmed by DNA sequencing. Afterwards, this plasmid was digested using EcoRI (New England BioLabs) and the resulting ZAB fragment was cloned into the suicide vector pEX18Tc (81), obtaining the pZAB7 plasmid, which was introduced by transformation into CC118λ*pir*. Selection was performed using tetracycline (4 μg/ml). pZAB7 was introduced by tripartite mating into *S. maltophilia* D457 (82), and selection was performed on LB agar plates with tetracycline (12 μg/ml) and imipenem (20 μg/ml). Tet^r colonies were streaked onto 12-μg/ml tetracycline plates and 10% sucrose plates. Tet^r and Sac^s colonies were streaked onto 10% sucrose plates and incubated at 30°C overnight. From the sucrose plates, Sac^r colonies were streaked onto 12-μg/ml tetracycline plates and 10% sucrose in order to obtain double recombinants with a partial deletion of the *smeZ* gene. Deletion was confirmed in the PBT100 strain using primers FragZAB_L (5′-GTGCAGAACCGGATCAAG-3′) and FragZAB_R (5′-CGAACTCGACAATGAGGAT-3′) and primers InternoSmeZ_F (5′-CGGTGTCGATCCTGTTCT-3′) and InternoSmeZ_R (5′-TGGATCGAGGTCATGAAATA-3′).

Determination of transient resistance to antibiotics. In order to determine the contribution of SmeVWX and SmeYZ to transient resistance to antibiotics, the respective inducer molecules of each efflux pump, sodium selenite and lincomycin, were chosen to measure bacterial growth in the presence of antibiotics. D457 and MBS704 (Δ*smeW*) strains were grown in ofloxacin (2 μg/ml), sodium selenite (0.5 mM), or ofloxacin and sodium selenite in combination at the same concentrations. D457 and PBT100 (Δ*smeZ*) strains were grown in the presence of amikacin (16 μg/ml), lincomycin (128 μg/ml), or amikacin and lincomycin in combination at the same concentrations. Ten-microliter volumes of overnight cultures of the three *S. maltophilia* strains were added to 140 μl of LB medium containing the different compounds to a final OD_{600} of 0.01 in 96-well plates (Nunclon Delta Surface). Plates were incubated at 37°C for 20 h using a Tecan Spark 10M plate reader (Tecan), and growth (OD_{600}) was recorded every 10 min. Plates were shaken for 5 s before every measurement.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01263-18.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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Biolog phenotype microarray: a tool for the identification of multidrug resistance efflux pumps inducers

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Supplemental material

Table S1. Biolog phenotype microarray tested compounds

PM11	PM12	PM13
Amikacin	Penicillin G	Ampicillin
Chlortetracycline	Tetracycline	Dequalinium chloride
Lincomycin	Carbenicillin	Nickel chloride
Amoxicillin	Oxacillin	Azlocillin
Cloxacillin	Penimepicycline	2, 2'-Dipyridyl
Lomefloxacin	Polymyxin B	Oxolinic acid
Bleomycin	Paromomycin	6-Mercaptopurine
Colistin	Vancomycin	Doxycycline
Minocycline	D, L-Serine hydroxamate	Potassium chromate
Capreomycin	Sisomicin	Cefuroxime
Demeclocycline	Sulfamethazine	5-Fluoroacil
Nafcillin	Novobiocin	Rolitetracycline
Cefazolin	2,4-Diamino-6,7-diisopropylpteridine	Cytosine-1-beta-D-arabinofuranoside
Enoxacin	Sulfadiazine	Geneticin (G418)
Nalidixic acid	Benzethonium chloride	Ruthenium red
Chloramphenicol	Tobramycin	Cesium chloride
Erythromycin	Sulfathiazole	Glycine

Neomycin	5-Fluorootic acid	Thallium (I) acetate
Ceftriaxone	Spectinomycin	Cobalt chloride
Gentamicin	Sulfamethoxazole	Manganese chloride
Potassium tellurite	L-Aspartic-β-hydroxamate	Trifluoperazine
Cephalothin	Spiramycin	Cupric chloride
Kanamycin	Rifampicin	Moxalactam
Ofloxacin	Dodecyltrimethylammonium bromide	Tylosin

PM14	**PM15**	**PM16**
Acriflavine	Procaine	Cefotaxime
Furaltadone	Guanidine hydrochloride	Phosphomycin
Sanguinarine	Cefmetazole	5-Chloro-7-iodo-8-hydroxyquinoline
9-Aminoacridine	D-Cycloserine	Norfloxacin
Fusaric acid	EDTA	Sulfanilamide
Sodium arsenate	5,7-Dichloro-8-hydroxyquinaldine	Trimethoprim
Boric acid	5,7-Dichloro-8-hydroxyquinoline	Dichlofluanid
1-Hydroxypyridine-2-thione	Fusidic acid	Protamine sulphate
Sodium cyanate	1,10-Phenanthroline	Cetylpyridinium chloride
Cadmium chloride	Phelomycin	1-Chloro-2,4-dinitrobenzene
Iodoacetate	Domiphen bromide	Diamide
Sodium dichromate	Nordihydroguaia retic acid	Cinoxacin
Cefoxitin	Alexidine	Streptomycin
Nitrofurantoin	5-Nitro-2-furaldehyde semicarbazone	5-Azacytidine
Sodium metaborate	Methyl viologen	Rifamycin SV

Chloramphenicol	3,4-Dimethoxy-benzyl alcohol	Potassium tellurite
Piperacillin	Oleandomycin	Sodium selenite
Sodium metavanadate	Puromycin	Aluminum sulphate
Chelerythrine	CCCP	Chromium chloride
Carbenicillin	Sodium azide	Ferric chloride
Sodium nitrite	Menadione	L-Glutamic-g-hydroxamate
EGTA	2-Nitroimidazole	Glycine hydroxamate
Promethazine	Hydroxyurea	Chloroxylenol
Sodium orthovanadate	Zinc chloride	Sorbic acid

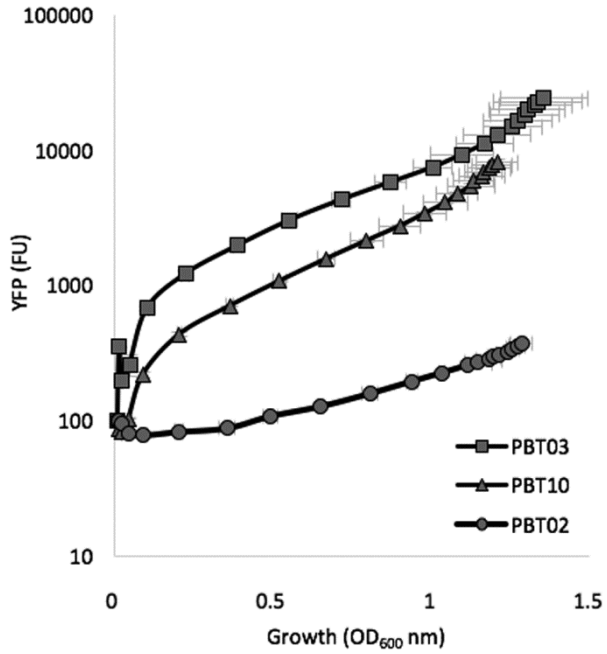

Figure S1. Growth and fluorescence values produced by sensor strains PBT03 (P_{EM7}), PBT10 (P_{YZ}) and PBT02 (P_{VWX}). Error bars show standard deviations from three independent replicates. FU, fluorescence units.

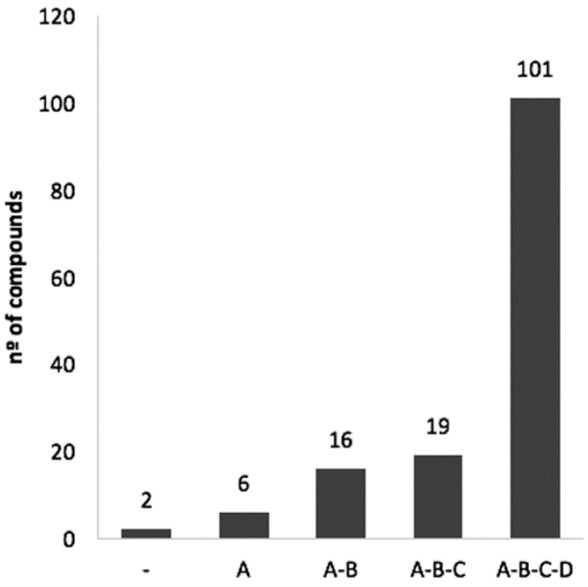

Figure S2. Compounds concentrations where *S. maltophilia* growth is detected. Compounds are divided in five categories. A-B-C-D compounds represent those agents where *S. maltophilia* growth is observable in all concentrations. A-B-C compounds are those where *S. maltophilia* grows only in A, B and C concentrations. The same reasoning for A-B and A compounds. No growth was observed for – compounds.

Article III

Involvement of the RND Efflux Pump Transporter SmeH in the Acquisition of Resistance to Ceftazidime in *Stenotrophomonas maltophilia*

P. Blanco, F. Corona, J. L. Martínez

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The low susceptibility to antibiotics that characterizes *S. maltophilia* limits the therapeutic options for the treatment of the infections that it causes. Although the combination treatment trimethoprim/sulfamethoxazole remains as the best drug of choice, other alternatives are also being evaluated. Among them, the use of ceftazidime, mainly in combination with other antibiotics, has been proposed as a treatment for *S. maltophilia* infections, although this treatment might be challenged due to the acquisition of resistance. Besides beta-lactamase overexpression, little information is available about other potential resistance mechanisms that might have an effect on *S. maltophilia* susceptibility to ceftazidime.

In the current work, we performed experimental evolution studies, followed by whole-genome sequencing, using increasing concentrations of ceftazidime with the purpose of identifying the genetic mechanisms and mutational trajectories involved in the acquisition of resistance to this beta-lactam drug. Among the identified mutations, we could determine a new mechanism in the acquisition of ceftazidime resistance: two amino acid substitutions in the transporter protein of the SmeGH RND efflux pump, SmeH, being the first report of mutations selected in an efflux pump transporter, and not in the efflux pump regulatory genes, in *S. maltophilia*. The analysis of the mutational trajectories shows that mutations in SmeH are the first step towards ceftazidime resistance in all the replicates. We also recreated both mutations in the wild-type strain D457, showing that they contribute to cross-resistance towards other beta-lactams as well, and that they do not compromise *S. maltophilia* fitness in an antibiotic-free medium. The structure prediction analysis of both amino acids residues in the transporter protein shows that the observed resistance phenotype can be due to changes in the access and binding pockets of the efflux pump transporter protein.

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Involvement of the RND efflux pump transporter SmeH in the acquisition of resistance to ceftazidime in *Stenotrophomonas maltophilia*

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The emergence of antibiotic resistant Gram-negative bacteria has become a serious global health issue. In this study, we have employed the intrinsically resistant opportunistic pathogen *Stenotrophomonas maltophilia* as a model to study the mechanisms involved in the acquisition of mutation-driven resistance to antibiotics. To this aim, laboratory experimental evolution studies, followed by whole-genome sequencing, were performed in the presence of the third-generation cephalosporin ceftazidime. Using this approach, we determined that exposure to increasing concentrations of ceftazidime selects high-level resistance in *S. maltophilia* through a novel mechanism: amino acid substitutions in SmeH, the transporter protein of the SmeGH RND efflux pump. The recreation of these mutants in a wild-type background demonstrated that, in addition to ceftazidime, the existence of these substitutions provides bacteria with cross-resistance to other beta-lactam drugs. This acquired resistance does not impose relevant fitness costs when bacteria grow in the absence of antibiotics. Structural prediction of both amino acid residues points that the observed resistance phenotype could be driven by changes in substrate access and recognition.

The evolution and dissemination of antibiotic resistance has become one of the major threats for public health worldwide, being the spread of multidrug resistant (MDR) Gram-negative bacteria one of the main problems nowadays[1]. *Stenotrophomonas maltophilia* is an opportunistic nosocomial pathogen responsible for causing a variety of infections, with high morbidity and mortality especially in patients with underlying pathologies, as cystic fibrosis, and in those who are immunocompromised[2–4]. One of the main characteristics of this bacterium is its low susceptibility to a broad range of antibiotics, which entails a difficulty in the treatment of the infections that it causes[5,6].

Among the elements that determine the intrinsic low susceptibility of *S. maltophilia* to antibiotics, it is important to highlight the low permeability of its membrane, as well as the presence in its genome of a number of intrinsic resistance genes that encode antibiotic-modifying enzymes, the quinolone resistance protein SmQnr and MDR efflux pumps, being of higher relevance those belonging to the resistance nodulation division (RND) family[7–11]. Few therapeutic options are currently in use for treating *S. maltohilia* infections. Among them, combinations of antibiotics including classical ones, as trimethoprim/sulfamethoxazole, are in use[12–15]. Despite *S. maltophilia* contains in its genome two intrinsic beta-lactamases, dubbed L1[9] and L2[10], they are not particularly active against ceftazidime at the level they are expressed in several clinical *S. maltophilia* isolates[16–18]. Consequently, use of ceftazidime, mainly in combination with other antibiotics, has been suggested for treating *S. maltophilia* infections[19–23], although the success of these treatments might be challenged given the large number of antibiotic resistance genes that *S. maltophilia* harbours. Indeed, it has been shown that mutants overexpressing L1 and L2 beta-lactamases and presenting a reduced susceptibility to ceftazidime can be easily selected *in vitro*[17,24]. Further, recent work has shown that *S. maltophilia* beta-lactamase-overexpressing ceftazidime-resistant mutants lacking a functional Mpl are frequently selected in clinics[25]. Despite these findings, little is still known about other potential

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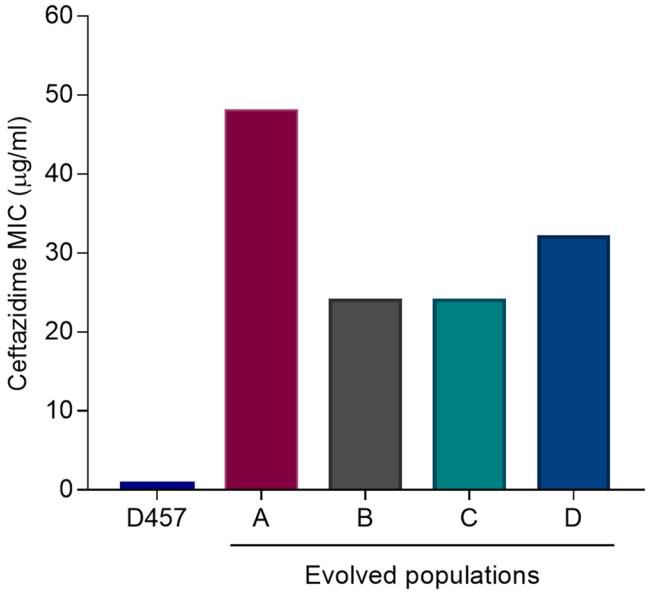

Figure 1. MICs for ceftazidime of the four *S. maltophilia* evolved populations. MIC was determined after 30 days of experimental evolution in the presence of increasing concentrations of ceftazidime in the populations A, B, C and D. The parental strain D457 was used as a reference. MIC, minimum inhibitory concentration.

resistance mechanisms, besides beta-lactamase overexpression, that might impact not only *S. maltophilia* susceptibility to ceftazidime, but to other antibiotics as well. To analyze this possibility, we challenged *S. maltophilia* with increasing concentrations of ceftazidime and analyzed the mutations that arised after this selection. Notably, we found that one of the first mutations to be selected was located in *smeH*, which encodes the transporter protein of the SmeGH RND efflux pump. RND efflux pumps are involved in the intrinsic resistance to several antibiotics when expressed at a basal level and their induction by environmental signals or conditions sensed by bacteria can lead to transient antibiotic resistance[26,27]. In addition, mutants overexpressing these efflux pumps are selected by antibiotics *in vitro* and are regularly isolated from infected patients[28–34].

Although some few publications address that mutations in the structural elements of efflux pumps can alter their specificity and hence contribute to antibiotic resistance[35–40], most works analyzing the role of efflux pumps in acquired antibiotic resistance focus on their level of expression[41–50], and not on the allelic variations in the genes encoding the efflux pump. Herein, we show that changes in the structure of SmeH, without the need of its overexpression, reduce the susceptibility of *S. maltophilia* to different antibiotics, including ceftazidime.

Results

Experimental evolution upon ceftazidime challenge leads to high levels of resistance in *S. maltophilia* populations. In order to elucidate how ceftazidime challenge impacts the acquisition of ceftazidime resistance, four independent *S. maltophilia* D457 cultures were serially passaged during 30 days of evolution in the presence of increasing concentrations of ceftazidime, reaching 32-fold the starting concentration at the end of the experiment. Four control cultures were also performed in the absence of antibiotic to track the selection of medium-adaptive mutations, not involved in resistance to ceftazidime challenge. Prior to the final evaluation of the evolved populations' susceptibility to ceftazidime, serial daily passages in LB medium without ceftazidime were performed for 3 days in order to exclude a possible induction of resistance caused by the previous ceftazidime exposure. Ceftazidime susceptibility was then evaluated in the four evolved populations (A, B, C and D) by Etest. As shown in Fig. 1, all the populations reached high levels of ceftazidime resistance after 30 days of evolution in comparison with the parental strain D457.

Selected mutations and temporal dynamics of *S. maltophilia* evolution in the presence of ceftazidime. With the aim of assessing the genetic mechanisms involved in ceftazidime resistance, the genomic DNAs of the evolved populations in presence of ceftazidime and the controls, grown in absence of antibiotics as well as of the parental strain, were extracted and sequenced. The coverage of the sequence was 106 on average, ranging from 90 to 113. The presence of mutations was determined in all populations as described in Methods, and confirmed by PCR amplification and Sanger sequencing. Only those mutations that were selected upon antibiotic selective pressure, but were absent in the populations evolved in the absence of selection, were taken into consideration. Table 1 shows those genetic changes that were identified after the whole genome sequencing (WGS) data analysis, and subsequently confirmed by Sanger sequencing, present in the ceftazidime-treated populations and absent in controls. In total, changes were found in 11 different genes. Most mutations were single nucleotide polymorphisms (SNPs) leading to amino acid substitutions. Among them, mutations in *smeH* were found in the four evolved populations, whereas mutations in *phoQ* were selected in three of them. Mutations in the penicillin binding protein (PBP) *ftsI* in population A, or in genes encoding for hypothetical proteins, as SMD_2719 also in population A, and SMD_0260 in population B, were also selected. Insertions or deletions

Population	Gene	Localization	Type	Nucleotide change	Amino acid change	Frequency (%)	Detected day of emergence
A	*smeH*	3061160	SNV	C → A	P326Q	98.7	5
	SMD_2719	3026930	SNV	A → C	V232G	51.6	20
	phoQ	315221	SNV	T → A	I76N	100	25
	ftsI	736289	SNV	C → A	A592D	100	25
	SMD_0534	613290	Ins	Ins of 1.037 bp	V398fs	—	30
B	*smeH*	3061160	SNV	C → A	P326Q	100	5
	smeH	3062171	SNV	A → G	Q663R	100	20
	SMD_0260	314100	SNV	A → G	K88R	70.1	25
C	*smeH*	3061160	SNV	C → A	P326Q	100	5
	yrbC	4744239	SNV	C → T	Q126*	32.3	30
	yciM	2035186	Del	Del of 188 bp	A206fs	—	30
	phoQ	315914	SNV	C → T	S307L	41.2	30
	phoQ	315965	SNV	C → T	P324L	37.5	30
D	*smeH*	3061160	SNV	C → A	P326Q	100	5
	smeH	3062171	SNV	A → G	Q663R	100	20
	phoQ	315236	SNV	C → T	P81L	99.2	20
	yrbE	4742678	Del	ATCGCCGTCG → —	I49fs	65.3	30
	SMD_1278	14224481	Ins	— →TGACTT	F91_G92insDF	57.1	30
	mrkC	685444	Del	GGCTTC → —	G187_F188del	76.3	30

Table 1. WGS-identified mutations in ceftazidime-evolved populations. SNV: single nucleotide variant; Ins: insertion; Del: deletion; Frequency (%): percentage of reads that contain the variation within a heterogeneous population; * STOP codon.

leading to frame shifts were also found in other genes. For instance, a 188-bp deletion in the lipopolysaccharide biosynthesis regulator *yciM* was detected in population C, and a 10-bp deletion in the permease component *yrbE* of an uncharacterized ABC transporter was selected in population D. In addition, an insertion sequence (IS) belonging to the IS5 family was also involved in the disruption of the gene *smd_0534*, which encodes a hypothetical protein, in population A. Noteworthy, and although every independent population seems to have a different resistance pattern, it was possible to find in the four antibiotic-evolved populations the same amino acid substitution (P326Q) in SmeH, the transporter protein of the SmeGH RND efflux pump.

The temporal dynamics of the different genetic changes were determined by amplifying and sequencing each of the mutated genes from stored samples of the four populations at days 5, 10, 15, 20, 25 and 30 (Table 1). The most remarkable aspect from these dynamics is the detection of the amino acid substitution P326Q in SmeH at day 5 of evolution in the four treated populations, when the ceftazidime concentration was 1 µg/ml. Further, in populations B and C, the following substitution in emerging was a second amino acid change in SmeH, Q663R, which was detected at day 20 when the ceftazidime concentration was 8 µg/ml. The remaining acquired genetic changes were found between days 20 and 30 of evolution.

Effect of SmeH substitutions on resistance and fitness. Since *smeH* mutations were found in the four ceftazidime-exposed populations and there are no previous studies regarding the contribution of the SmeGH efflux pump to antibiotic resistance, we decided to determine the role of both P326Q and Q663R substitutions in *S. maltophilia* ceftazidime resistance. In order to do this, both mutations were recreated in the parental strain D457, either together or separately, obtaining the strains PBT101 (P326Q), PBT102 (Q663R) and PBT103 (P326Q; Q663R). The MIC of ceftazidime was determined for the three recreated strains, as well as for the parental strain D457. While P326Q substitution was able to increase more than 5-fold the ceftazidime MIC (4 µg/ml), the second substitution Q663R did not lead to a change in the susceptibility by itself (0.75 µg/ml). Nevertheless, the presence of both mutations led to a 10-fold change in the MIC levels (8 µg/ml).

To explore whether the ceftazidime resistance mutations in *smeH* lead to cross-resistance and/or collateral sensitivity to other antibiotics, changes in the susceptibility to several classes of antibiotics were measured in the strains PBT101, PBT102 and PBT103 using the parental strain D457 as a reference. The MICs of the different antibiotics are shown in Supplementary Table S1. As observed in Fig. 2, a two-fold increase in the MIC was obtained for the beta-lactams cefazolin and aztreonam in the PBT101 strain. In the case of the PBT103 strain, the fold change for these antibiotics was 2-fold and 6-fold, respectively. Further, cross-resistance in this strain was also observed for cefotaxime (2-fold change) and the quinolone norfloxacin (3-fold change). As happened with ceftazidime, no changes in the MICs for beta-lactams were observed in the PBT102 strain; however, a 4-fold change in the MIC was obtained for tetracycline. No collateral sensitivity was observed for any of the tested antibiotics.

In order to study in more detail the observed resistance to beta-lactams in the PBT101 and PBT103 strains, a more sensitive method based on bacterial growth was applied. The OD_{600} was recorded in the presence of ceftazidime, cefotaxime and cefoxitin for 20 h. Strains D457, PBT102 and PBT104 were also included in the experiment. Data shown in Fig. 3B, C and D, proved that only mutants PBT101 and PBT103 were able to grow in the presence of the three selected antibiotics, being the resistance of the PBT103 strain higher than that of PBT101. Meanwhile,

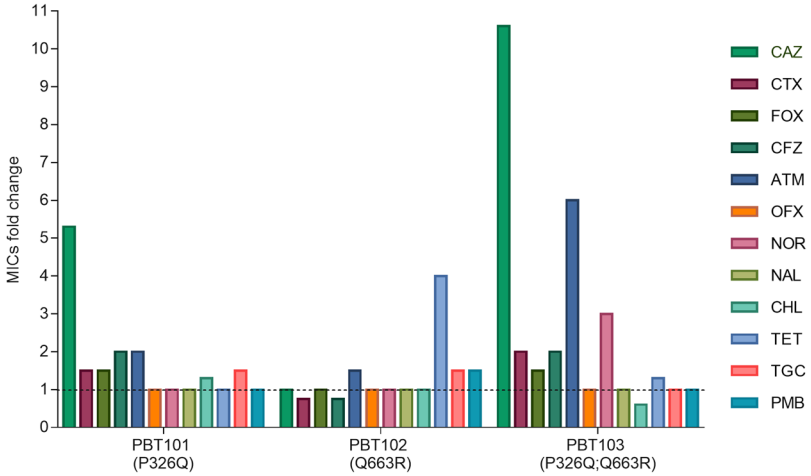

Figure 2. Susceptibility of the *S. maltophilia* mutants to different antibiotics. MICs were measured in the three recreated mutants PBT101 (P326Q), PBT102 (Q663R) and PBT103 (P326Q; Q663R). Fold changes were determined using the MIC values of the wild-type strain D457 as reference (dotted line). MIC, minimum inhibitory concentration; CAZ, ceftazidime; CTX, cefotaxime; FOX, cefoxitin; CFZ, cefazolin; ATM, aztreonam; OFX, ofloxacin; NOR, norfloxacin; NAL, nalidixic acid; CHL, chloramphenicol; TET, tetracycline; TGC, tigecycline; PMB, polymyxin B.

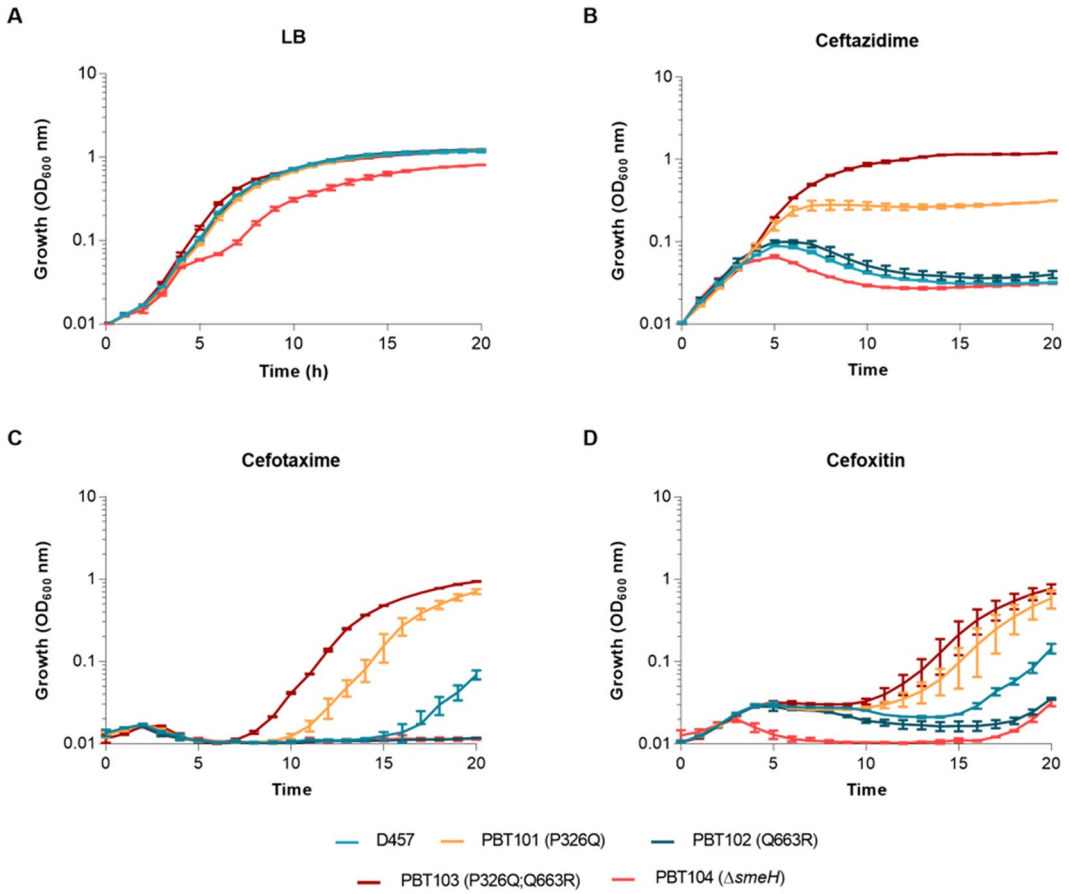

Figure 3. Effect of beta-lactams on the growth of *S. maltophilia* strains. Growth curves were performed for the three *S. maltophilia* recreated mutants PBT101 (P326Q), PBT102 (Q663R) and PBT103 (P326Q; Q663R), the *smeH*-defective strain PBT104 and the parental strain D457 in the absence (**A**) or presence of ceftazidime (4 µg/ml) (**B**), cefotaxime (256 µg/ml) (**C**) and cefoxitin (128 µg/ml) (**D**) during 20 h. Error bars show standard deviations from three independent experiments.

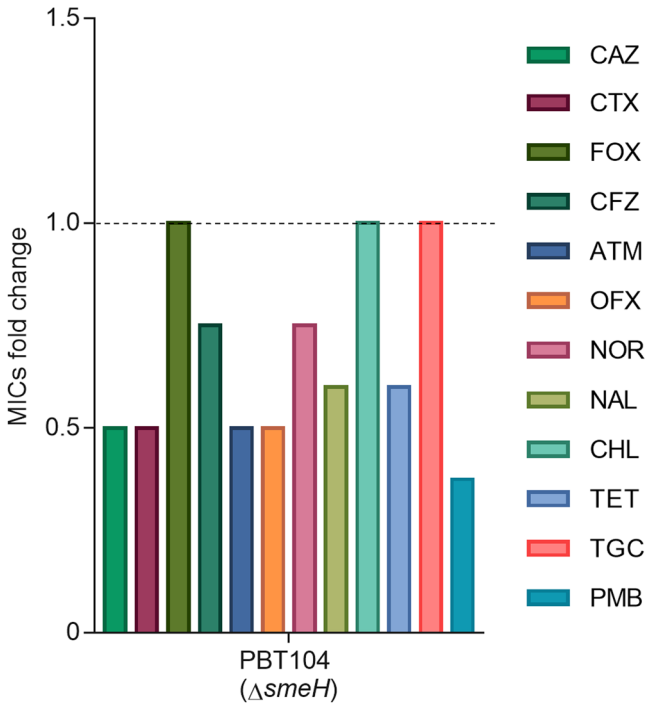

Figure 4. Susceptibility of the *S. maltophilia smeH*-defective mutant to different antibiotics. MICs were measured in the PBT104 strain (*ΔsmeH*) and the fold changes were determined using the MIC values of the wild-type strain D457 as reference (dotted line). MIC, minimum inhibitory concentration; CAZ, ceftazidime; CTX, cefotaxime; FOX, cefoxitin; CFZ, cefazolin; ATM, aztreonam; OFX, ofloxacin; NOR, norfloxacin; NAL, nalidixic acid; CHL, chloramphenicol; TET, tetracycline; TGC, tigecycline; PMB, polymyxin B.

the PBT102 mutant displayed a growth similar to the observed for the wild-type strain in all conditions, confirming the above-obtained MIC results. We also evaluated the effect of these mutations on fitness cost. As shown in Fig. 3A, the strains PBT101, PBT102 and PBT103 had no growth defect in comparison with the parental strain in LB medium; only the PBT104 strain, which lacks *smeH*, showed a slight growth impairment. These results point that fitness is not compromised in an antibiotic-free medium when *smeH* mutations are present.

Role of SmeGH efflux pump in the intrinsic resistance and the physiology of *S. maltophilia*. Besides studying its contribution to the acquisition of resistance, we decided to assess the role of the SmeGH efflux pump in the *S. maltophilia* intrinsic resistance through the generation of a *smeH*-deficient mutant. In addition, we wanted to address whether the original substrates of the wild-type allele of the efflux pump are the same as those of the selected mutants and hence the amino acid substitutions just modify the efficiency; or they are different and the mutations alter the substrates specificity of SmeH. The MICs for ceftazidime, as well as for the other antibiotics, were determined in the PBT104 (*ΔsmeH*) strain and are shown in Supplementary Table S1. As shown in Fig. 4, the *smeH*-deficient mutant PBT104 showed an increased susceptibility to ceftazidime and to almost all tested antibiotics, excepting chloramphenicol and tigecycline, in comparison with the wild-type strain. In the case of cefoxitin, no changes in the MIC were observed; however, as shown in Fig. 3D, the PBT104 mutant presents a more impaired growth, as compared to the wild-type strain, in the presence of this beta-lactam. These results suggest that these antibiotics could be substrates of the SmeGH efflux pump, which would then be involved in *S. maltophilia* intrinsic resistance.

In addition to commonly-used antibiotics, we evaluated the implication of the SmeGH efflux pump in the susceptibility to other antimicrobial toxic compounds. As shown in Table 2, the *smeH*-deficient strain PBT104 was more susceptible than the parental strain to the oxidative stress-generating compounds menadione and tert-butyl hydroperoxide, as well as to the biocide benzalkonium chloride and to the plant-derived compound naringenin. Changes in the MICs were also observed for some of the compounds in the *smeH*-mutant alleles selected in presence of ceftazidime. A two-fold increase in the MIC was observed in the PBT101 (P326Q) strain for all the tested biocides (hexachlorophene, benzalkonium chloride and triclosan). Further, a higher susceptibility to menadione was detected in the PBT102 (Q663R) and PBT103 (P326Q; Q663R) strains, and a lower MIC was also obtained for tert-butyl hydroperoxide in the PBT103 strain. These data suggest that, besides its contribution to antibiotics resistance, SmeGH might be involved in the detoxification of some other toxic compounds and the amino acid substitutions in SmeH selected in presence of ceftazidime could have different effects on the capability of this efflux pump for extruding other compounds.

The role of SmeGH in *S. maltophilia* physiology was also studied through the evaluation of virulence-related characteristics, as biofilm formation and swimming motility. As shown in Fig. 5A, the PBT104 (*ΔsmeH*) strain

Strain	MD (mM)	TBHP (mM)	NGEN (mM)	HCP (μg/ml)	BAC (μg/ml)	TRI (μg/ml)
D457	1.25	0.62	25	2	6.25	25
PBT101 (P326Q)	1.25	0.62	25	4	12.5	50
PBT102 (Q663R)	0.62	0.62	25	2	6.25	25
PBT103 (P326Q; Q663R)	0.50	0.31	25	2	6.25	25
PBT104 (*ΔsmeH*)	0.12	0.31	3.12	2	1.56	25

Table 2. MICs of antimicrobial compounds for *S. maltophilia* strains. MIC, minimum inhibitory concentration; MD, menadione; TBHP, tert-butyl hydroperoxide; NGEN, naringenin; HCP, hexachlorophene; BAC, benzalkonium chloride; TRI, triclosan.

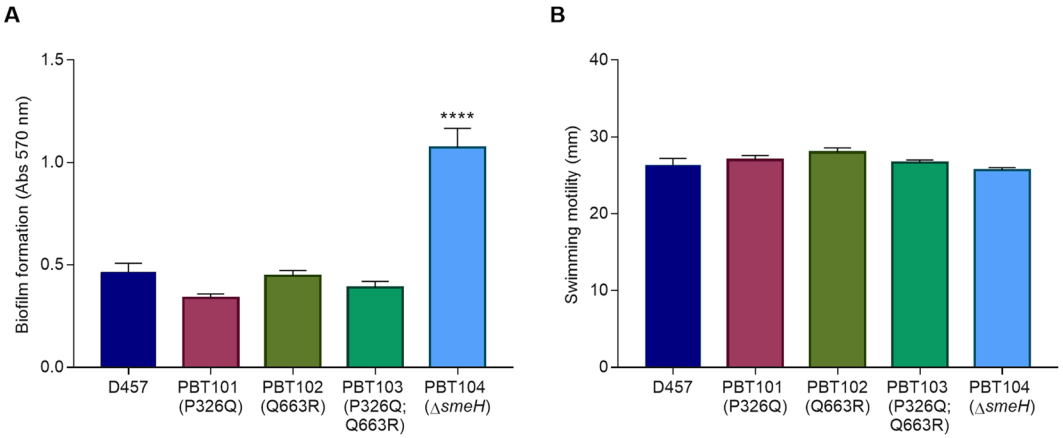

Figure 5. Biofilm formation and swimming motility of *S. maltophilia* strains. (**A**) The biofilm formation assay was carried out by measuring the absorbance at 570 nm of crystal violet after 48 h incubation. Error bars show standard deviations from eight replicate samples. ****Indicates $P < 0.001$ calculated by one-way ANOVA test. (**B**) The swimming motility assay was performed by measuring the growth area after 48 h incubation on LB semisolid agar (0.3%) plates. Error bars show standard deviations from three independent experiments.

presented an enhanced ability to form biofilm in comparison with the parental strain D457, while no significant differences in the swimming ability were detected among the different strains (Fig. 5B).

Ceftazidime-selected SmeH substitutions are likely located in the vicinity of the access and deep binding pockets of the transporter protein. With the purpose of elucidating the molecular basis behind the phenotype conferred by both SmeH mutations, a structural alignment of the *S. maltophilia* SmeH transporter was performed with the *Escherichia coli* AcrB efflux protein sequence, showing that both proteins are homologous (Template modeling score 0.97, sequence identity 0.51, coverage 0.98). The alignment showed that residue P326 is conserved in both transporter proteins (Supplementary Fig. S1); consequently, the available crystal structure of AcrB (PDB ID code 4DX7.B[37]) was used for predicting the effect of the mutation P326Q in *S. maltophilia*. It has been described that AcrB monomers can adopt one of three conformations, labeled as loose (L), tight (T) and open (O), depending on the step of the drug export process (access, binding, and extrusion, respectively)[51]. Using these templates, the P326 residue was predicted to be located in the proximities of the deep binding pocket in the tight (T), or binding, monomer of AcrB (Fig. 6A). Q663, which corresponds to the V672 in AcrB, was also represented in the AcrB crystal structure (PDB ID code 4DX7.A[37]), and it was mapped at the bottom of the access pocket of the efflux protein (Fig. 6B).

Since P326 is conserved in both species, we wanted to study the effect of the P326Q substitution in the AcrB crystal in its doxorubicin-bound form (PDB ID code 4DX7.B[37]) in order to have a reference of the deep binding pocket conformation when a known substrate is bound inside. In its wild-type form, the protamine residue is mapped under the deep binding pocket of the transporter protein, but contactless (Fig. 7A). However, when Q326 is represented, the glutamine residue can be found pointing into the deep binding pocket cavity (Fig. 7B). Although in this simulation the glutamine residue does not seem to interact with the cocrystallized substrate directly, it might establish contact with other residues that are part of the binding pocket and change in some way the affinity for the antibiotic.

Discussion

Experimental evolution studies provide important information on the genetic adaptive changes underlying the acquisition of different phenotypes by bacterial populations[52], including antibiotic resistance[53]. In the current work, we have used such approach for analyzing the evolutionary trajectories of the relevant nosocomial pathogen *S. maltophilia* towards ceftazidime resistance. As it happens in the case of other studies on evolution towards

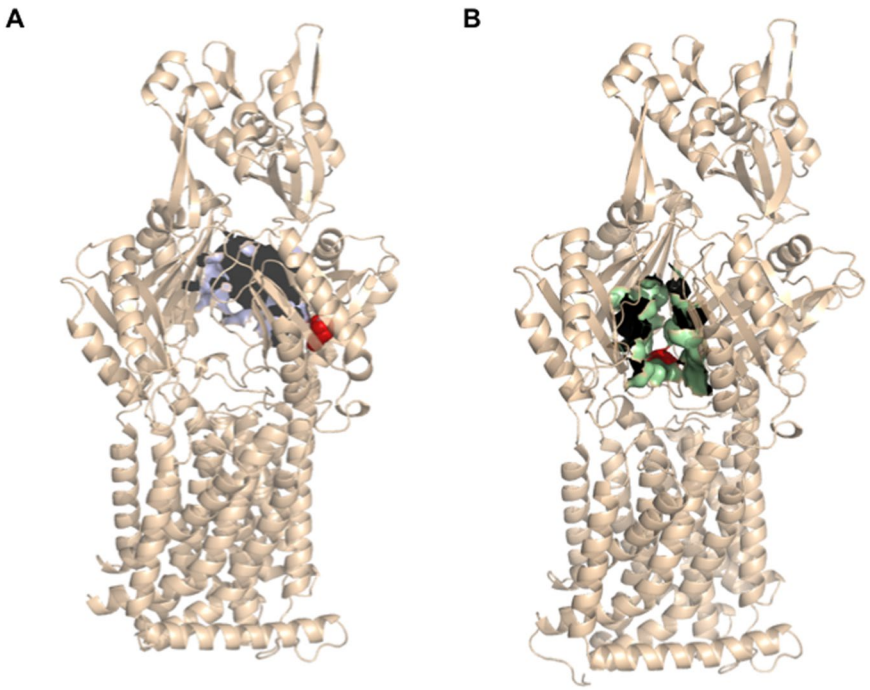

Figure 6. Mapping of SmeH-amino acid residues on the 3D structure of AcrB. (**A**) Surface representation of the predicted deep binding pocket (violet) on the AcrB tight monomer (PDB code 4DX7.B). P326 is represented by red spheres. (**B**) Surface representation of the predicted access pocket (green) on the AcrB loose monomer (PDB code 4DX7.A). V672, the Q663-corresponding residue in AcrB, is represented by red spheres.

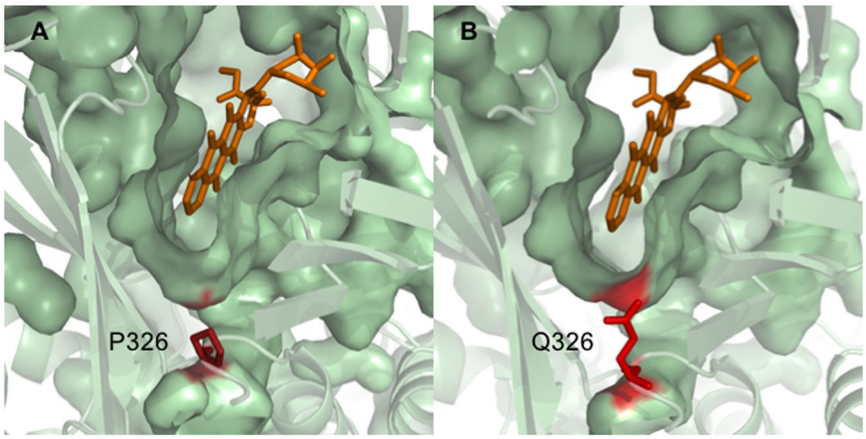

Figure 7. Effect of P326Q substitution on the residue orientation in the SmeH deep binding pocket. Surface representation of the deep binding pocket on the AcrB tight monomer (PDB code 4DX7.B) with the wild-type residue P326 (**A**) and the mutant allele Q326 (**B**). Both residues are represented by red sticks and doxorubicin is represented in orange.

antibiotic resistance[54], the evolutionary trajectories followed by *S. maltophilia* parallel cultures submitted to antibiotic selective pressure, shared some common elements, namely mutations in the efflux pump transporter gene *smeH* (present in the four evolved populations) and in the two-component sensor histidine kinase *phoQ* (three out of the four evolved populations), indicating that, as stated in[54,55], evolution towards antibiotic resistance might present some degree of predictability. Some genes that present mutations in the evolved bacterial populations have been previously described to have a role in antimicrobial resistance in other microorganisms. This is the case of *ftsI*, a gene encoding a peptidoglycan transpeptidase[56], whose mutation has been reported to be involved in resistance to beta-lactam drugs[55,57,58], or *phoQ*, which is involved in polymyxin and antimicrobial peptides resistance[59,60], although its potential role in beta-lactams resistance has not been studied in detail. The other genes presenting mutations involved in the acquisition of ceftazidime resistance in *S. maltophilia* have not been described previously to be involved in antibiotic resistance. Namely, mutations in genes encoding the auxiliary

(*yrbC*) and the permease components (*yrbE*) of an uncharacterized ABC transporter, or the RND transporter *smeH*, as well as four hypothetical proteins were selected upon antibiotic selective pressure. Temporal analysis of the dynamics of evolution showed that several of the mutations emerged from day 20 of evolution; however, for all the populations, the first step leading to acquisition of resistance to ceftazidime was the P326Q substitution in SmeH, being the first and only mutation detected at day 5 of evolution. A second substitution in SmeH, Q663R, emerged in two of the treated populations days later after P326Q.

Considering the important role that the SmeGH efflux pump seems to have in the acquisition of ceftazidime resistance, we focused on these amino acid substitutions to get more insights into their contribution to resistance. Data showed that strains PBT101 (P326Q) and PBT103 (P326Q; Q663R) exhibited an increase of 5- and 10-fold in the ceftazidime MIC, respectively, while PBT102 (Q663R) remained at the same level of the wild-type strain, indicating this mutation to be neutral concerning ceftazidime resistance in the wild-type genomic background. Meanwhile, the presence of both mutations in the *smeH* gene has a positive effect regarding ceftazidime resistance, having the second mutation an impact just in combination with the first one. In agreement with data derived from the study of the evolution of extended spectrum beta-lactamases[61], this result supports the notion that epistatic interactions (including neutral mutations) modulate the evolution towards resistance and, consequently, the order of acquisition of mutations is highly relevant for the final phenotypic outcome.

Several RND efflux pumps extrude a broad range of unrelated compounds from the bacterial cell[62]. With the aim of determining whether the P326Q and Q663R changes are specific for ceftazidime resistance or they change *S. maltophilia* susceptibility to other antibiotics, MICs of other drugs were measured. Our results showed that PBT101 exhibited increased resistance to the beta-lactams aztreonam and cefazolin, and PBT103, besides these ones, presented also increased resistance to cefotaxime and the quinolone norfloxacin. Besides, growth-based experiments allowed us to detect an increased resistance against cefotaxime in the PBT101 strain, and against cefoxitin in both PBT101 and PBT103 strains. These data indicate that P326Q and the combination of both P326Q/Q663R in SmeH do not confer specific resistance to ceftazidime, but also to other antibiotics, mostly beta-lactams. Conversely, PBT102 did not show any change in the susceptibility to beta-lactams but it did for tetracycline, suggesting that, while the Q663R substitution does not play any role in beta-lactams resistance by itself, it could lead to tetracycline resistance, a feature that may allow to select this mutation in a wild-type background, independently of the P326Q mutation, under tetracycline challenge.

Finally, the hyper-susceptibility of the *smeH*-defective mutant PBT104 to some beta-lactam drugs, as well as to quinolones, polymyxin B and tetracycline, suggests that SmeGH is an intrinsic resistant determinant for these antibiotics. In addition, the fact that this mutant presents changes in the susceptibility to the same beta-lactams as PBT101 and PBT103 does indicate that the mutations selected in *smeH* along experimental evolution likely alter the efficiency of extrusion of these substrates.

According to our results, SmeGH might not only be involved in resistance to antibiotics, but also to other toxic compounds belonging to different families, as the oxidative stress agents menadione and tert-butyl hydroperoxide, the plant-derived compound naringenin, and the biocide benzalkonium chloride. Other *S. maltophilia* RND efflux pumps have been previously reported to be involved in the extrusion of toxic molecules. For instance, SmeDEF, besides antibiotics, is able to extrude triclosan[63] and the plant exudate phloretin[64]. Since RND efflux pumps are important bacteria detoxification elements[65], it is possible that some of these compounds are substrates of SmeGH, which would participate in their detoxification. Further, as happened with beta-lactams, PBT101, PBT102 and PBT103 strains also showed changes in their MICs to some of these toxic compounds, indicating that the amino acid substitutions present in SmeH in these mutants and selected in presence of ceftazidime could interfere in the capability of this efflux pump for extruding these molecules.

In order to get more insights into the physiological roles that the SmeGH efflux pump could have, biofilm formation and swimming motility assays were assessed in all the strains. While the swimming ability was neither compromised nor increased in any of the *S. maltophilia* strains, deletion of the transporter component SmeH led to an enhanced biofilm production in the PBT104 strain. Although the molecular mechanisms regarding this phenotype remain to be clarified, it is possible that the SmeGH efflux pump contributes negatively to biofilm formation by the regulation of biofilm-related genes, as happens with the ABC transporter lm.G_1771 in *Listeria monocytogenes*[66], or by the accumulation of toxic molecules that could trigger the expression of genes involved in biofilm formation through a general stress response, as proposed by Bazzini *et al*. in the case of *Burkholderia cenocepacia*[67].

It has been generally assumed that, in the absence of selection, the acquisition of antibiotic resistance will impose a burden to the resistant organism, which would be outcompeted by the susceptible ones[68–70]. For instance, mutations leading to the overproduction of MDR efflux pumps can lead to the extrusion of beneficial compounds outside the cell, as well as a non-physiological expense of energy, having an impact on the bacterial fitness unless compensation mechanisms are triggered[71] or secondary compensatory mutations are selected[72]. With the aim of determining the effect on the *S. maltophilia* fitness of each of the *smeH* mutations alone and in combination, all the strains were grown in absence of antibiotics and their growth was recorded. The only strain impaired in growth was the *smeH*-deficient PBT104, while none of the resistant mutants showed any deficiency regarding bacterial growth, indicating that: I) the removal/inhibition of intrinsic resistance determinants may impair bacterial fitness. In other words, intrinsic resistance determinants might be under positive selection pressure; II) the presence of the analyzed antibiotic resistance mutations, separate or together, does not lead to a decrease on the *S. maltophilia* fitness in an antibiotic-free environment; and III) the second mutation in *smeH* does not seem to have been selected for compensating the potential fitness cost of the first mutation, at least in the recreated strains background. Previous studies have shown that some resistant mutations are cost-free[73–75], or even confer an enhanced fitness and virulence in infected hosts[76,77]. This fact makes that bacteria harbouring no-cost resistance mutations, as those described in the current article, to be more likely to persist in the absence of antibiotics[78].

Acquisition of resistance in *S. maltophilia* regarding RND efflux pumps, is usually associated with mutations in their regulatory genes[28–30]. Nevertheless, this is the first report of mutations occurring in an efflux pump transporter protein that lead to a change in the susceptibility to several antibiotics belonging to different categories in this bacterium. Previous studies have shown that amino acid substitutions in the transporter proteins of RND efflux pumps belonging to other bacterial species can change the susceptibility to some antibiotics[35–40]. In these reports, authors highlight the importance of those substitutions located inside or near the transporter binding pocket, where they are of relevance for the recognition and/or accommodation of certain substrates. To better understand how the amino acid substitutions in SmeH impact in the observed acquisition of resistance in *S. maltophilia*, we mapped both residues P326 and Q663 using the AcrB structure from *E. coli* as a model, since no crystal structures for SmeH or other *S. maltophilia* transporters are available. The location of both residues suggests that they could be involved in the entrance and/or recognition of the SmeH substrates, which would have an effect in the resistance levels to these compounds. Since the P326 residue is conserved in both proteins, we performed an *in silico* mutagenesis on the AcrB structure in its doxorubicin-bound form in order to determine whether the mutation P326Q had an impact in the deep binding pocket conformation. According to our prediction, this amino acid change made the Q326 residue to be pointing to the base of the deep binding pocket. In addition to AcrB, P326 is also found to be conserved in the AcrB-homolog MexB from *P. aeruginosa* (Supplementary Fig. S1), as well as in another RND transporter protein from the same bacterium, MexD, corresponding in this case with the P328 moiety. Mao *et al.*[79] reported that, together with other amino acid substitutions, P328L in MexD increased resistance in *P. aeruginosa* against aztreonam and carbenicillin. This residue was mapped to the large periplasmic loop (LPL) of the RND efflux pump, which can potentially surround the drug-binding pocket. In agreement with this, LPLs have also been reported to play an important role in substrate recognition in AcrB and AcrD from *E. coli*[80]. Although it is unclear how this mutation affects the efflux pump function, the change in the residue orientation might cause interactions with other neighboring residues that are part of the cavity and influence somehow the recognition of the efflux pumps substrates, as beta-lactams, leading to the observed resistance phenotype.

But, why does Q663R only confer changes in beta-lactams susceptibility in combination with P326Q, and not by itself? It has been recently reported that V672, the Q663-equivalent residue in AcrB, might form part of the meropenem binding site of this protein[81]. Besides, our mapping prediction showed that this residue was located at the bottom of the AcrB access pocket. We postulate that changes just in the access cavity could not be enough to have a direct effect on substrate extrusion; however, when P326Q is present and, presumably, binding is ameliorated, the existence of Q663R suppose a beneficial outcome, since both access and recognition of the antibiotic would be improved. Further studies on the structures of RND efflux pump transporters might shed light on this issue.

Concluding Remarks

Experimental evolution and whole-genome sequencing after ceftazidime exposure, have revealed new mechanisms of acquired resistance in the opportunistic pathogen *S. maltophilia*. Among them, we highlight the selection, in all the ceftazidime-challenged strains, of mutations in the RND efflux pump transporter *smeH* for the first time. Some few publications have reported that mutations in efflux transporters may decrease the susceptibility to antibiotics of bacterial pathogens[35–40]. However, this is the first formal demonstration showing that this type of mutations can be selected as a first response to the presence of antibiotics. Besides contributing to ceftazidime resistance, the presence of P326Q and P326Q/Q663R also confers resistance to other beta-lactam drugs and do not suppose a fitness cost in an antibiotic-free environment. The SmeH-homolog from *E. coli* AcrB has been a suitable model for predicting the positions of both amino acid residues, showing that P326 is located in the proximities of the deep binding pocket of the efflux pump, while V672, the corresponding Q663 in AcrB, was mapped at the bottom of the access pocket. Taken together, these results suggest that changes in both access and binding pockets might be required for the incremented observed resistance. Whether or not *in vitro* experimental evolution assays are of relevance for understanding evolution during infection in the treated patient is a topic that requires further studies. Nevertheless, a recent analysis of the evolution of carbapenem resistance in *Acinetobacter baumannii* during a prolonged infection in a burn patient shows that a first event in the evolution was the selection of a mutation in a gene encoding a structural component of an efflux pump (*adeB*), followed by the mutation of *ftsI*[82]. This evolution trajectory resembles the one we have observed and supports that the application of experimental evolution and whole-genome sequencing approaches can help to understand and identify the mechanisms of acquired resistance to ceftazidime and other antibiotics by bacterial pathogens.

Materials and Methods

Bacterial strains and growth conditions. All the plasmids and strains used in this work are listed in Table 3. The wild-type strain *S. maltophilia* D457 was used as the parental strain for the evolution experiments. Ceftazidime (GlaxoSmithKline) was used at different concentrations during the evolution assay. Cultures were grown using LB medium at 37 °C. Antibiotics were added when required: ampicillin (Ap; 100 μg/ml) for *E. coli* containing the pGEM-T Easy and pGEM-T derived plasmids. Medium was supplemented with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 80 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in order to induce and detect beta-galactosidase production.

Experimental evolution. Experimental evolution was performed with the wild-type strain D457 growing in the presence of increasing concentrations of ceftazidime. Cultures were initially grown at the maximum ceftazidime concentration that allowed growth (1 μg/ml) in liquid LB medium. One microliter of a bacterial overnight culture was inoculated in four independent test tubes containing 2 ml of LB medium with ceftazidime Serial passages were performed inoculating 1 μl of bacterial cell cultures in fresh medium containing the same

Strain or plasmid	Description	Source or reference
Bacterial strains		
E. coli		
OmniMAX	Strain used in transformation. F' *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80(*lacZ*)ΔM15 Δ(*lac*ZYA-argF) U169 *end*A1 *sup*E44 *thi*−1 *gyr*A96 *rel*A1 *deo*R *ton*A *pan*D	Invitrogen, Life Technologies
CC118λpir	Donor cell in conjugation. Strain CC118 lisogenized with λ *pir* phage (Tc') Δ(*ara-leu*), *ara*D, Δ*lac*X74, *gal*E, *gal*K, *pho*A20, *thi*-1, *rps*E, *rpo*B, *arg*E (Am), *rec*A1	[84]
1047 (pRK2013)	Helper cell in conjugation harbouring pRK2013 (Kan^R) plasmid	[87]
S. maltophilia		
D457	Clinical strain	[88]
PBT101	D457 carrying the *smeH* P326Q substitution	This work
PBT102	D457 carrying the *smeH* Q663R substitution	This work
PBT103	D457 carrying the *smeH* P326Q and Q663R substitutions	This work
PBT104	D457 Δ*smeH*	This work
Plasmids		
pGEM®-T Easy Vector	Cloning vector, Amp^R	Promega
pPBT21	pGEM-T-derived plasmid carrying the fragment SmeH:P326R	This work
pPBT22	pGEM-T-derived plasmid carrying the fragment SmeH:Q663R	This work
pEX18Tc	Gene replacement vector; *sacB*, Tet^R	[83]
pPBT101	pEX18Tc-derived plasmid carrying the fragment SmeH:P326Q	This work
pPBT102	pEX18Tc-derived plasmid carrying the fragment SmeH:Q663R	This work
pHAB5	pEX18Tc-derived plasmid containing the 5′ and 3′ regions of *smeH*	This work

Table 3. Bacterial strains and plasmids.

antibiotic concentration every 24 h for 4 days. At day 5, ceftazidime concentration was doubled. This procedure was repeated for 30 days (around 300 generations), until the ceftazidime concentration was 32 μg/ml (32-fold increase). Four independent replicates were also cultured in the same conditions, but in the absence of antibiotic as controls. Every 5 days, and after the passage, the minimum inhibitory concentration (MIC) for ceftazidime was determined for all the evolving populations and samples were taken and kept at −80 °C for further analysis.

DNA extraction, WGS and identification of mutations. After the 30-days experimental evolution, the total genomic DNAs from the evolved populations and the original wild-type D457 strain were extracted using a Gnome® DNA kit following the manufacturer's protocol (MP Biomedicals). Quality and quantity of the extracted DNA was assessed by agarose gel electrophoresis and using a NanoDrop Spectrophotometer (Thermo Fischer), respectively. DNA samples were sent to the CRG (Center for Genomic Regulation, Barcelona) and sequenced using the HiSeq2,000 Sequencing System (Illumina) generating 125-bp paired-end reads. The number of reads per sample was 2,093,625 on average, representing a sequencing depth of 100x approximately. Data analysis was accomplished with CLC Genomics Workbench software (Qiagen) and the resulting reads were mapped to the *S. maltophilia* D457 reference genome (NC_017671.1) using default parameters. Single nucleotide polymorphisms (SNPs) detection was performed using the Fixed Ploidy Variant Detection tool (ploidy = 1, required variant probability = 90%, minimum coverage = 8, minimum frequency = 15%) and the given variants were filtered against those obtained for the wild-type D457 strain. Insertions, deletions, inversions, tandem duplications and translocations were detected using the InDel and Structural Variants tool (P-value threshold = 0.0001). The identified mutations were verified by PCR and Sanger-sequencing. Unless otherwise stated, the thermocycler was programmed for 56 °C of annealing. Primers are listed in Supplementary Table S2.

Recreation of the *smeH* mutations. The amino acid substitutions P326Q and Q663R were introduced alone and in combination in *S. maltophilia* D457. Two 1,000-bp regions of the *smeH* gene, containing each one SNP, were amplified from the ceftazidime evolved population B using primers SmeH_snp1_F and SmeH_snp1_R for Q326 amplification, and SmeH_snp2_F and SmeH_snp2_R for R663 amplification. Both 1,000-bp fragments were cloned into pGEM-T Easy (Promega) following the manufacturer's instructions obtaining respectively pPBT21 and pPBT22 plasmids, which were introduced by transformation into *E. coli* OmniMAX (Invitrogen). Constructions were verified by DNA sequencing. Both plasmids were extracted with the Qiaprep ® Spin Miniprep Kit 250 (Qiagen) following the manufacturer's protocol, and digested with EcoRI (New England BioLabs). The obtained products containing either Q326 or R663 were purified and cloned into the suicide vector pEx18Tc[83], giving rise to pPBT101 and pPBT102, respectively. Both plasmids were then introduced by transformation into CC118*λpir* and selection was performed using tetracycline (4 μg/ml). Then, by tripartite matting, pPBT101 or pPBT102 were introduced into *S. maltophilia* D457[84] and selection was carried out on LB agar plates containing tetracycline (12 μg/ml) and imipenem (20 μg/ml). Tet^R colonies were then streaked onto LB agar plates with 10% sucrose and 12 μg/ml tetracycline. Tet^R and Sac^S colonies were selected and streaked onto LB plates with 10% sucrose. From these sucrose plates, Sac^S colonies were streaked onto LB plates containing sucrose 10% and 12 μg/ml tetracycline with the aim of obtaining the mutants with the amino acid changes. The presence of the

mutations was confirmed by SNP-specific PCR; the P326Q change was identified in the PBT101 strain using primers Comp_snp1_F and Comp_snp1_R; the Q663R modification was confirmed in the PBT102 strain using primers Comp_snp2_F and Comp_snp2_R (65 °C annealing temperature). DNA sequencing was performed with primers SmeH_snp1_F and SmeH_snp1_R, and SmeH_snp2_F and SmeH_snp2_R to further confirm the presence of the mutations. Plasmid pPBT102 was introduced as described above in the PBT101 strain in order to obtain a mutant with both amino acid substitutions. Confirmation was carried out in the PBT103 strain with the above-mentioned primers and DNA sequencing. Primers are listed in Supplementary Table S2.

Generation of a *smeH* deletion mutant. A derivative *S. maltophilia* mutant with a partial deletion in the *smeH* gene was generated through homologous recombination. Using primers HAF and HAR (Table S1), a 494-bp fragment (HA) corresponding to the 5′-end of *smeH* was amplified; primers HBF and HBR were used to amplify a 514-bp fragment (HB) of the *smeH* 3′-end. The thermocycler was programmed for 10 cycles of 52 °C of annealing, followed by 20 cycles of 56 °C. Using HA and HB fragments as templates, an overlapping PCR was carried out using primers HAF and HBR, yielding a 1,000-bp fragment (HAB). The obtained product was cloned into pGEM-T Easy (Promega) and introduced by transformation into *E. coli* CC118*λpir*. DNA sequencing was performed for sequence verification. The plasmid was then digested with EcoRI and the HAB fragment was cloned into pEx18Tc. The resulting plasmid pHAB5 was introduced by transformation into *E. coli* CC118*λpir* and then, by tripartite matting, into *S. maltophilia* D457. The *smeH*-defective mutant was selected following the above-described process and confirmation of the deletion in the PBT104 strain was performed using the primers Ext_smeH_L and Ext_smeH_R, as well as Int_smeH_L and Int_smeH_R. Primers are listed in Supplementary Table S2.

Antimicrobials susceptibility assay. MICs for the antibiotics cefotaxime, cefoxitin and cefazolin, as well as for menadione, tert-butyl hydroperoxide, hexachlorophene, benzalkonium chloride, triclosan and naringenin, were determined by microbroth double dilution in 96-well microtiter plates (NUNCLON™ Delta Surface) containing LB medium with two-fold dilutions of each antibiotic. Two replicates of each strain were inoculated to a final OD_{600} of 0.01 and plates were incubated for 20 h at 37 °C without shaking. MICs for ceftazidime, aztreonam, ofloxacin, norfloxacin, nalidixic acid, chloramphenicol, tetracycline, tigecycline and polymyxin B were determined using MIC test strips (Liofilchem), which were placed on LB agar plate seeded with an overnight 1:1,000 dilution of each bacterial population. Plates were incubated at 37 °C and results were analyzed after 20 h. The MIC was defined at the lowest concentration at which no bacterial growth was observed.

Growth curves in the presence of β-lactams. The *S. maltophilia* wild-type strain D457 and the derived mutants PBT101 (SmeH:P326Q), PBT102 (SmeH:Q663R), PBT103 (SmeH:P326Q;Q663R), and PBT104 (Δ*smeH*) were grown in the presence or in the absence of ceftazidime (4 µg/ml), cefoxitin (256 µg/ml) or cefotaxime (128 µg/ml). The assay was carried out in 96-well plates (NUNCLON™ Δ Surface) where 10 µl of an overnight bacterial culture were added to 140 µl of LB medium, with or without the antibiotics, to a final OD_{600} of 0.01. Plates were then incubated at 37 °C for 20 h, and growth (OD_{600}) was measured every 10 min using the plate reader Tecan Spark 10 M (Tecan). Shaking for 5 s was performed every 10 min before each measurement.

Biofilm formation assay. An overnight culture from each tested strain was diluted 1:100 and 100 µl of bacterial suspension were inoculated per well in a 96-well plate (Costar Serocluster™, Corning Incorporated). After 48 h incubation at 37 °C without agitation, biofilms were stained by adding 25 µl of crystal violet 0.1% for 5 min. The stained biofilms were rinsed three times using 100 µl of Milli-Q water and then 150 µl of 0.25% Triton X-100 were added in order to dissociate biofilms. Hundred microliters were transfer to a clean 96-well plate (Nunclon™ Delta Surface) and biofilm formation was assessed through the quantification of crystal violet staining by measuring absorbance at 570 nm. The assay was performed in octuplicate.

Swimming assay. The swimming motility of all the tested strains was determined on LB agar (0.3%) plates. An overnight culture from each strain was diluted to a final OD_{600} of 2 and 5 µl were spotted on the surface of the swimming plates. After 48 h incubation at 30 °C, the growth zone was measured in milliliters. The assay was performed in triplicate.

Prediction of the amino acid substitutions location. Structural alignment of SmeH and AcrB amino acid sequences was performed using I-Tasser[85]. the positions of the SmeH amino acid substitutions were represented using the AcrB crystal structures of *E. coli* as a reference (PDB ID codes 4DX7.A and 4DX7.B[37]). Prediction of both access and deep binding pocket cavities in AcrB was performed with CASTp[86]. Figures were generated using PyMol (The PyMOL Molecular Graphics System, Schrödinger, LLC).

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Author Contributions

P.B. contributed to the design of the study and performance of the experiments. F.C. contributed to the design of the study. J.L.M. contributed to the design of the study. All authors contributed to results interpretation and to the writing and reviewing of the article.

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Involvement of the RND efflux pump transporter SmeH in the acquisition of resistance to ceftazidime in *Stenotrophomonas maltophilia*

Paula Blanco, Fernando Corona, José Luis Martínez

Supplementary information.

Table S1. MICs of several antibiotics for *S. maltophilia* strains

	MIC (μg/ml)											
Strain	**CAZ**	**CTX**	**FOX**	**CFZ**	**ATM**	**OFX**	**NOR**	**NAL**	**CHL**	**TET**	**TGC**	**PMB**
D457	0.75	128	256	4,096	4	1	8	6	6	1.5	0.5	8
PBT101 (P326Q)	4	192	384	8,192	8	1	8	6	8	1.5	0.75	8
PBT102 (Q663R)	0.75	96	256	3,072	6	1	8	6	6	6	0.75	12
PBT103 (P326Q; Q663R)	8	256	384	8,192	24	1	24	6	4	2	0.5	8
PBT104 (*ΔsmeH*)	0.38	96	256	3,072	2	0.5	6	4	6	1	0.5	3

MIC, minimum inhibitory concentration; CAZ, ceftazidime; CTX, cefotaxime; FOX, cefoxitin; CFZ, cefazolin; ATM, aztreonam; OFX, ofloxacin; NOR, norfloxacin; NAL, nalidixic acid; CHL, chloramphenicol; TET, tetracycline; TGC, tigecycline; PMB, polymyxin B.

Table S2. Primers used in this study

Name	Sequence (5'-3')	Utilization
SmeH_snp1_F	GAATTCAGTACGCCATGAACATCTGG	Amplification of the 1000-bp fragment containing P326Q in *smeH*
SmeH_snp1_R	GAATTCACTTGTTGAAGGTGCGGTAG	
SmeH_snp2_F	GAATTCCCGGTGAGATCTACAAGCAG	Amplification of the 1000-bp fragment containing Q663R in *smeH*
SmeH_snp2_R	GAATTCGACTTGACCACCGTGTTGAG	
Comp_snp1_F	GTGACCTGGTTCTCGCA	Verification of Q326
Comp_snp1_R	GAACAGGGTCAGCTGGTT	
Comp_snp2_F	ACCTGCCCACCGTGCG	Verification of R663
Comp_snp2_R	CGGCCTTCGTAGAAGAAGTCGTT	
HAF	GAATTCCGAACATCCAGTCTTCGCCTG GGTGGTTGCGATCCTG	Amplification of the 489-bp fragment corresponding to the 5'-end of *smeH*
HAR	AGCTTTCATACAGGGCGGCCGGGGAT ACGCGAGACCTGGT	
HBF	ACCAGGTCTCGCGTATCCCCGGCCGCC CTGTATGAAAGCT	Amplification of the 489-bp fragment corresponding to the 3'-end of *smeH*
HBR	GAATTCTCAACGATCCGGGCGGTGTG CAGCATCCGC	
Ext_smeH_L	GATTCGAACAAGCAGTAAG	Amplification of the 3404-bp fragment including the complete *smeH* gene
Ext_smeH_R	ATTGGTAATCGTGGCAGTGT	
Int_smeH_L	ACAACTACGGCTTCGACAC	Amplification of a 211-bp fragment inside s*meH* gene
Int_smeH_R	GTCTTGACCACTTCCTGGAT	
acrD1_L	GTCGGCCAGCCAGGTACT	Verification of P326Q in *smeH*
acrD1_R	ATCTGGGTCATCGCCTTCT	

acrD2_L	ATGATCCTGTTCGTGGTGCT	Verification of Q663R in *smeH*
acrD2_R	GAAGCTCTTCAGCGACTCCT	
phoQ_cef_L	ACCCTGGCGATAACACGAT	Verification of I76N and P81L in *phoQ*
phoQ_cef_R	ACCTTGGTCAACTCGGTGAT	
phoQ_tig3_L	CGAATTCCCGTACACCATCT	Verification of S307L and P324L in *phoQ*
phoQ_tig3_R	CACTTGAAGGCGTTCTCCAG	
mrkC_L	GCTGGCACCTACCGTGTC	Verification of G187_F188del in *mrkC*
mrkC_R	AGATCAGCTGCTGGTTCTGG	
ftsI4_L	CAGATCATCGACGAGAACG	Verification of A592D in *ftsI*
ftsI4_R	AACATCGAAGGACTCATTGC	
yrbE_L	GTTCTCGCTGACCGTGCT	Verification of I49fs in *yrbE*
yrbE_R	GTGAACAACATTGCCGACAG	
yrbC_L	GTCGGCAAGTACATGTTCAG	Verification of Q126* in *yrbC*
yrbC_R	AAGTGCGTTACTTGCCATTG	
yciM_L	ATCGAAGATGGATTTCGTCA	Verification of A206fs in *yciM*
yciM_R	AGGTCAGCAACGCACTGA	
SMD_0534_L	TGCGGAATTTTTCGAGTATC	Verification of V398fs in *smd_0534*
SMD_0534_tig_R	AGGTTGGAGATGGACTGTGG	
SMD_1278_L	CCCAACCACTCTTCTGTGG	Verification of F91_G92insDF in *smd_1278*
SMD_1278_R	CGTTCCTGGTACTCCTTGAC	
SMD_0260_L	GATTGGAACCTGGTCGAG	Verification of K88R in *smd_0260*
SMD_0260_R	TCTTCGACCAGAAGGATACG	

SMD_2719_L	GCAACGGGATCTGGGT	Verification of V232G in *smd_2719*
SMD_2719_R	ACCACGAACGGCAGCTTG	

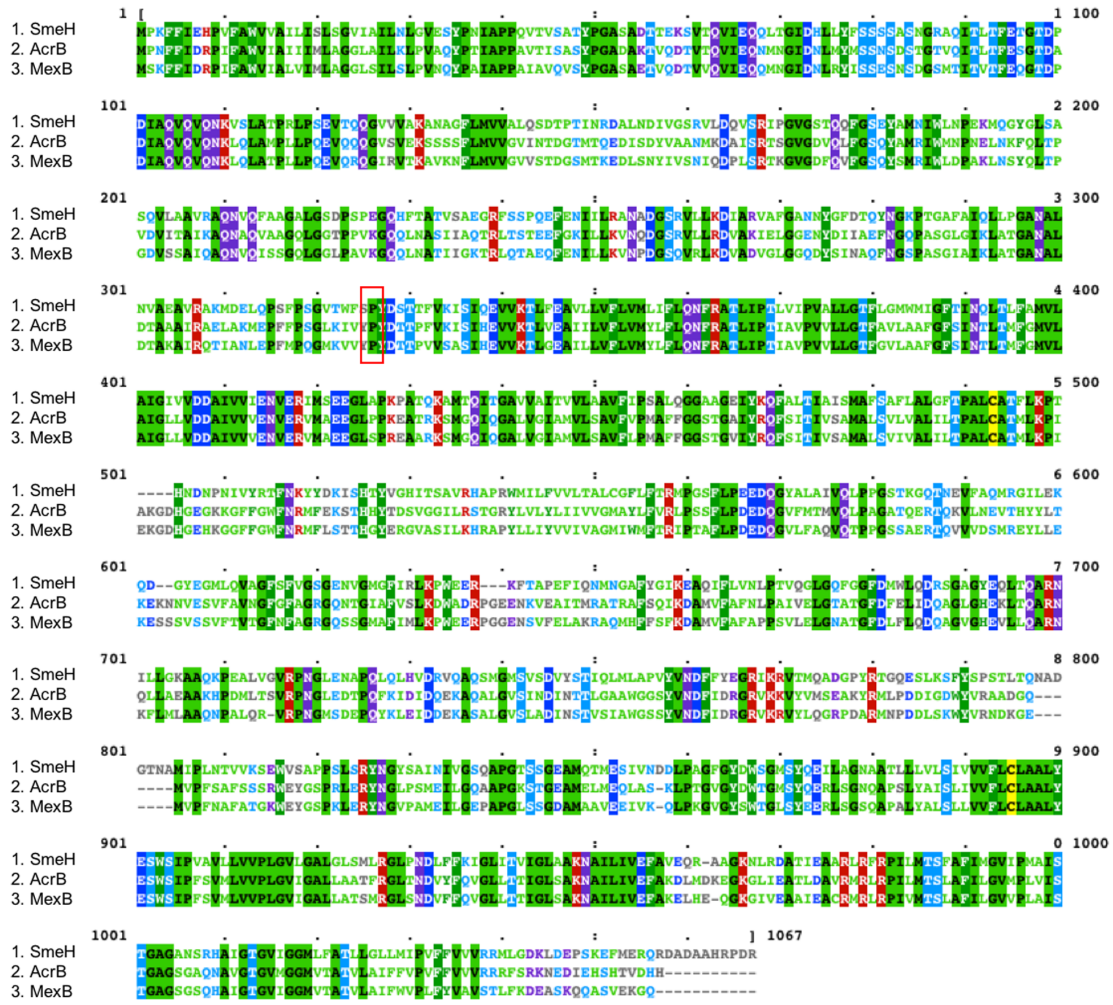

Figure S1. Alignment of the *S. maltophilia* SmeH amino acid sequence with its homologues AcrB from *E. coli*, and MexB from *P. aeruginosa*. The conserved P326 residue is marked with a red square in the amino acid sequences of the three species. Color scheme is based on amino acid identity.

Article IV

Mechanisms and Phenotypic Consequences of Tigecycline Resistance Acquisition in *Stenotrophomonas maltophilia*

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Journal of Antimicrobial Chemotherapy. In press

Despite the fact that trimethoprim/sulfamethoxazole (SXT) is the first-line agent for the treatment of *S. maltophilia* infections, the increased acquisition of resistance to this antibiotic by this bacterial species has complicated its use. In addition to the beta-lactam ceftazidime, the glycylcyclines-group antibiotic tigecycline, has shown a good activity against SXT-resistant *S. maltophilia* isolates. Some of the molecular mechanism involved in the acquisition of tigecycline resistance have been studied in other bacterial species. However, concerning *S. maltophilia*, little is known about the mechanisms involved in the acquisition of mutation-driven resistance to this antibiotic.

In the present work, we challenged *S. maltophilia* with increasing concentrations of tigecycline and then analysed the mutations by whole-genome sequencing that were potentially involved in the acquisition of resistance to this drug. Besides acquiring high-level resistance at the end of the experimental evolutions, all the replicates acquired mutations in the gene encoding the transcriptional regulator of the SmeDEF RND efflux pump, SmeT, being the first event towards tigecycline resistance. In addition, mutations affecting the ribosome biogenesis and the lipopolysaccharide biosynthesis pathway, were also detected. The effect of these mutations on the susceptibilities against other antibiotics were also assessed, showing that the tigecycline-evolved populations display cross-resistance to several antibiotics, notably quinolones, tetracycline, chloramphenicol or aztreonam, but are hypersusceptible to fosfomycin. Finally, all the evolved populations, as well as single isolated clones, present a significant fitness cost when they grow in the absence of antibiotic. This effect could compromise the maintenance of these mutants in the population when the selection pressure is absent.

Mechanisms and phenotypic consequences of acquisition of tigecycline resistance by *Stenotrophomonas maltophilia*

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Running title: Predicting *S. maltophilia* tigecycline resistance.

Synopsis: The full understanding of the mechanisms leading to the acquisition of antibiotic resistance by bacterial pathogens requires the identification of the mutational pathways that make bacteria become resistant to antibiotics. In the present work, we use adaptive laboratory evolution and whole-genome sequencing with the aim of exploring the genetic mechanism involved in the acquisition of tigecycline resistance by the multidrug-resistant opportunistic pathogen *Stenotrophomonas maltophilia*. Our study shows that all the evolved populations are able to reach high-level resistance against tigecycline following different mutational trajectories, yet with some common elements. Among the mechanism involved in the low susceptibility to tigecycline, mutations in the SmeDEF efflux pump negative regulator *smeT*, changes in proteins involved in the biogenesis of the ribosome, and modifications in the lipopolysaccharide biosynthesis pathway, seem to play a major role. Besides tigecycline resistance, the evolved populations present cross-resistance to other antibiotics, as aztreonam and quinolones, and they are hypersusceptible to fosfomycin. Whether or not the use of fosfomycin sequentially or in combination with tygecycline would be useful for reducing the emergence of *S. maltophilia* antibiotic resistant mutants is a feature that remains to be established. However, it is clear that sequential use of tygecycline and quinolones should be disregarded. Further, we found that the selected resistance mechanisms impose a relevant fitness cost when bacteria grow in the absence of antibiotic, suggesting that mutational resistance to tigecycline should be easily selected along treatment, but will not be stably maintained in *S. maltophilia* populations.

Introduction

Stenotrophomonas maltophilia is an opportunistic nosocomial pathogen causing different infections, mainly in immunocompromised patients and in those with underlying pathologies. [1,2] Further, this microorganism is one of the most common pathogens causing chronic lung infections in cystic fibrosis patients. [3,4] *S. maltophilia* exhibits a characteristic phenotype of low susceptibility to antibiotics, being considered as a model of intrinsically resistant pathogen. [5] Besides presenting a low membrane permeability, *S. maltophilia* possesses a set of intrinsic resistance determinants, including antibiotic-modifying enzymes and multidrug resistance (MDR) efflux pumps. [6-9] Further, acquisition of antibiotic resistance by mutation is particularly relevant when this bacterium produces a chronic infection. [10]

The antimicrobial options for the treatment of *S. maltophilia* infections are limited, being trimethoprim/sulfamethoxazole (SXT) the most effective drug of choice. [11] However, different mechanisms of resistance, such as the presence of *sul1*, *sul2* and *dfrA* genes, and the increased expression of MDR efflux pumps, limits its use. [12-15] Tigecycline, which presents good *in vitro* activity against SXT-resistant strains isolates, [16-18] is a promising alternative for treating *S. maltophilia* infections. Tigecycline belongs to a new group of tetracyclines called glycylcyclines, whose target is the ribosomal A site of the 30S subunit, leading to translation interference. [19,20] The main mechanism of tigecycline resistance in pathogens as *Klebsiella pneumoniae*, *Bacteroides fragilis*, *Acinetobacter baumannii*, *Enterobacter* spp or *Salmonella enterica* is the overexpression of MDR efflux pumps. [21] The presence of the flavin-dependent monooxygenase TetX, which modifies first- and second-generation tetracyclines, has also been described as a tigecycline resistance mechanism in *Bacteroides* strains. [22] In *Escherichia coli*, besides overexpression of the AcrAB efflux pump, tigecycline resistance has also been associated with mutations affecting the lipopolysaccharide (LPS) biosynthesis pathway. [23] Regarding *S. maltophilia*, the tigecycline resistance mechanisms are not well established. A recent study has proposed that the observed non-susceptibility to tigecycline of a set of *S. maltophilia* clinical isolates might be due to the overexpression of the resistance nodulation division (RND) SmeDEF efflux pump. [24] However, this statement is based in correlations and a formal demonstration of the molecular basis of the acquisition of tigecycline resistance by *S. maltophilia* is still needed.

To this end, experimental evolution studies in the presence of increasing concentrations of tigecycline were performed in *S. maltophilia*. This approach, followed by whole-genome sequencing (WGS), has allowed to determine the genetic changes underlying antibiotic resistance, as well as the evolutionary trajectories towards that resistance in different organisms. [25,26] Besides providing a predictive analysis of the potential genetic determinants involved in tigecycline resistance, the cross-resistance and collateral sensitivity against other antibiotics, as well as the resistance-associated fitness costs, were also assessed.

Materials and Methods

Growth conditions and antibiotic susceptibility assays

Bacteria were grown using liquid Lysogeny Broth, Lennox (LB) at 37 °C with agitation at 250 rpm. Evolution experiments were performed with *S. maltophilia* D457. Tigecycline (Pfizer) was used at different concentrations during the evolution period. The minimum inhibitory concentrations (MICs) for tigecycline, ciprofloxacin, ofloxacin and erythromycin were determined by double dilution in 96-well microtiter plates (NUNCLON™ Delta Surface) in LB at 37 °C. MICs of ceftazidime, aztreonam, trimethoprim/sulfamethoxazole, chloramphenicol, fosfomycin, nalidixic acid, streptomycin, gentamicin and tetracycline were determined using MIC test strips (Liofilchem) in LB-Agar plates at 37 °C.

Experimental evolution

Eight independent experimental evolutions (four in the presence of tigecycline and four controls without antibiotic) were performed during 30 days. Tigecycline-challenged *S. maltophilia* D457 populations were initially grown at the maximum concentration that allowed bacterial growth in these conditions (1 mg/L) and serial passages were performed as described in Blanco *et al*. [27] until a final concentration of 32 mg/L was reached at day 30. Samples were preserved at -80 °C every 5 days for further analysis.

Whole-genome sequencing (WGS)

Genomic DNA was extracted from the evolved populations at day 30 of evolution, as well as from the D457 parental strain, using a Gnome® DNA kit (MP Biomedicals). The quality and quantity of the DNA was determined by agarose gel electrophoresis and by using a NanoDrop spectrophotometer, respectively. WGS was performed at the Center for Genomic Regulation (CRG, Barcelona) using a HiSeq 2,000 Sequencing System (Illumina), which generated 125-bp paired-end reads. On average, the number of reads per sample was 2,399,250, which represents a sequencing coverage of 100x approximately.

Data analysis and verification of genetic changes

The *S. maltophilia* D457 reference genome (NC_017671.1) was used to align the WGS-obtained reads. Mutations were identified using the CLC Genomics Workbench software (Qiagen). Single nucleotide polymorphisms (SNPs) were analyzed using the Fixed Ploidy Variant Detection tool and the resulting variants were filtered against those obtained for the parental D457 strain. Other deviations from the reference genomes, such as inversions, insertions, deletions or duplications were identified using the InDel and Structural Variants tools. PCR amplification of the regions containing the mutations and Sanger-sequencing were performed to verify the WGS-detected mutations. The primers used are shown in Supplementary Table S1.

Fitness cost determination

Bacterial samples from the four tigecycline-evolved populations, as well as single clones isolated from each population at different time points of the evolution, were used for the assay. Ten µl of each culture were inoculated in 140 µl of LB medium to a final OD_{600} of 0.01 using 96-well plates (NUNCLON^{TM} Δ Surface). Growth (OD_{600}) from three independent replicates was monitored every 10 min using the plate reader Spark 10M (Tecan) during 20 h at 37 ºC. Shaking for 5 s was performed before each measurement. OD_{600} values at exponential growth phase were used to calculate the maximum growth rates. Relative growth rates were calculated by dividing the values of each population, or individual clone, to those obtained for the parental strain D457 from the same experiment.

Results

Step-wise evolution of *S. maltophilia* towards tigecycline resistance

Four independent *S. maltophilia* D457 lineages were serially passaged for 30 days in the presence of increasing concentrations of tigecycline until reaching 32 mg/L. In parallel, four independent replicates were also passaged at the same conditions in the absence of antibiotic as control. At the end of the experiment, the antibiotic-evolved cultures were submitted to three daily passages in LB medium in order to discard any potential induction of resistance triggered by tigecycline exposure. Afterwards, MICs for the four tigecycline-evolved populations were determined. As shown in Figure 1, all the populations exhibit high-level resistance against tigecycline, ranging from 96 to 192 mg/L.

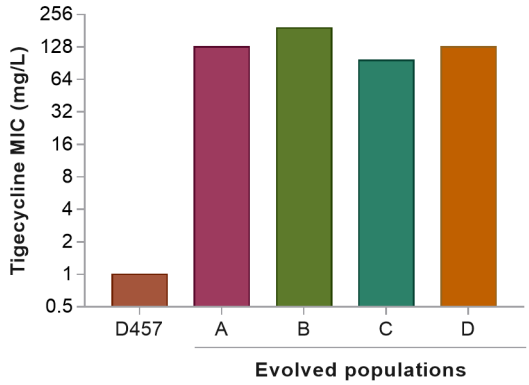

Figure 1. MICs of tigecycline of the four *S. maltophilia* evolved populations. Tigecycline susceptibility was determined in populations A, B, C and D after 30 days of experimental evolution in the presence of increasing concentrations of tigecycline. The wild-type strain D457 was used as a reference. MIC, minimum inhibitory concentration.

Mutations and adaptive trajectories to tigecycline resistance in *S. maltophilia*

Whole genomic DNA of all the evolved populations, as well as that of the parental strain D457, were sequenced in order to determine the genetic changes potentially responsible for the tigecycline decreased susceptibility. Those mutations selected under tigecycline pressure, but absent in the control populations evolved in the absence of antibiotic, were taken into consideration and are listed in Table 1. Altogether, genetic modifications were found in 19 different genes, being SNPs causing amino acid substitutions the most common changes. The four antibiotic-evolved populations shared some genetic changes on common elements. A mutation in *smeT,* which in *S. maltophilia* encodes the repressor of the RND efflux pump SmeDEF, [28] was found in all the populations. In addition, the four populations present mutations in the ribosome recycling protein *frr*. SNPs in other ribosome-related genes, such as in *rpsU* and *rpsJ* in populations A and B, respectively, and a single-base insertion in *rpsA* in population C, were also detected. Genes encoding different enzymes were also found mutated, including those involved in the LPS biosynthesis, such as the phosphoethanolamine transferase *SMD_RS16325* in population A, the lipid A biosynthesis lauroyl acyltransferase *htrB* in population B, and the UDP-glucose dehydrogenase *ugd* in population D; or involved in the phosphatidic acid biosynthetic process, as *dgkA* in population D. In addition to SNPs, insertions or deletions leading to frame shifts in the protein-coding sequences were also detected. For instance, a 30-bp

insertion was found in the *rpoD* gene in population C, and a 101-bp deletion was identified in the TIGR02099 family protein coding gene (*SMD_RS15850)* in population D. Two different inverted duplications were also detected. The first one was found in population C and comprised 262 genes (~ 300 kbp), while the second one was found in population D and included 53 genes (~ 60 kbp), which are common to those of the duplicated-inverted sequence in population C (Figure 2; Supplementary Table S2).

Table 1. WGS-identified mutations in the tigecycline-evolved lineages

P	Gene	Product	Localization	Type	Nucleotide change	Amino acid change	(%)	Detected day of emergence
A	*smeT*	TetR-family transcriptional regulator	4099641	SNV	T → A	L166Q	100	5
	frr	Ribosome recycling protein	1493591	SNV	T → G	T50P	94.9	10
	SMD_RS 16325	Phosphoethanolamine transferase	3492054	SNV	C → T	S284L	99.2	15
	rpsU	30S ribosomal protein S21	434736	SNV	C → T	E24K	78.7	30
	SMD_RS 15850	TIGR02099 family protein	3391847	Ins	Ins-GCCGCCA	V239fs	54.5	ND
B	*smeT*	TetR-family transcriptional regulator	4099641	SNV	T → A	L166Q	100	5
	frr	Ribosome recycling protein	1493470	SNV	G → T	P90Q	100	15
	rpsJ	30S ribosomal protein S10	900713	SNV	T → G	V73G	100	15
	speD	S-adenosylmethionine decarboxylase proenzyme	4369955	SNV	A → G	V117A	100	15
	htrB	Lipid A biosynthesis lauroyl acyltransferase	3952269	SNV	A → G	H290R	100	30
	SMD_RS 02920	Hypothetical protein	613184	SNV	C → T	Q364*	100	30
C	*smeT*	TetR-family transcriptional regulator	4099733	SNV	A → C	T197P	99.1	5
	frr	Ribosome recycling protein	1493591	SNV	T → G	T50P	98.5	15

	phoQ	Two-component sensor histidine kinase	315823	SNV	G → T	V277L	98.4	15
	hutI	Imidazolonepropionase	2992107	SNV	G → A	G283D	99.2	15
	InvDup	-	1331537	-	-	-	ND	20
	dsbB	Periplasmic thiol:disulfide oxidoreductase	915108	SNV	C → T	A20V	70.2	25
	ompR	Two-component system regulatory protein	3753106	Ins	Ins-T	L54fs	32.3	30
	rpsA	30S ribosomal protein S1	2033510	Ins	Ins-C	N463fs	49.6	30
	dedD	Cell division protein	950264	SNV	C → G	A249G	26.4	ND
	dedD		950269	SNV	A → T	S251C	25.2	ND
	rpoD	RNA polymerase sigma factor	4232255	Ins	Ins-30 bp	A176_D177insEVIVGFNDLA	ND	ND
D	*smeT*	TetR-family transcriptional regulator	4099641	SNV	T → A	L166Q	100	5
	cheY	Chemotaxis protein	3488433	SNV	C → T	E21K	100	10
	frr	Ribosome recycling protein	1493591	SNV	T → G	T50P	98.9	15
	ugd	UDP-glucose dehydrogenase	3075488	SNV	A → G	F66S	100	20
	dgkA	Diacylglycerol kinase	796027	Ins	Ins-TGCTGG	L71_A72insVL	48.4	30
	SMD_RS15850	TIGR02099 family protein	3391974	Del	Del-101 bp	V163fs	ND	ND
	InvDup	-	1425560	-	-	-	ND	ND

P: Population; SNV: single nucleotide variant; Ins: insertion; Del: deletion; fs: frame shift; InvDup: inverted duplication; (%): Frequency, percentage of reads that contain the variation within a heterogeneous population; ND: non-determined (CLC Genomics Software do not determine the frequency of big insertion/deletions; ND in the mutation day of emergence was due to low frequency of the mutation in the population or impairment of sequence amplification.

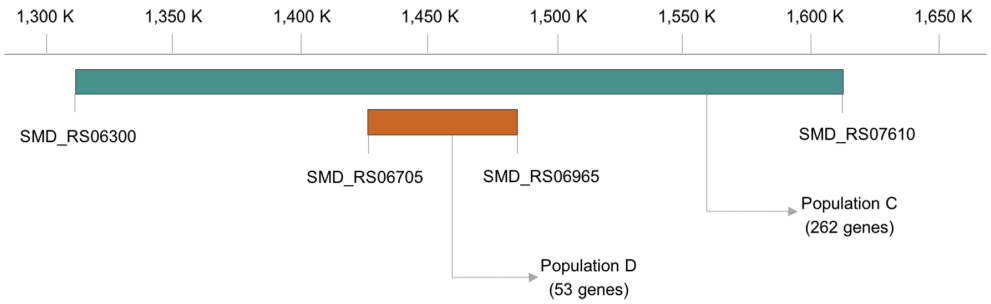

Figure 2. Inverted duplications detected in the tigecycline-evolved populations C and D. Two different inverted duplicated sequences were detected after 30 days of tigecycline evolution in populations C (~300 kbp) and D (~60 kbp), containing 262 and 53 genes, respectively.

In order to confirm the presence of the WGS-identified genetic changes and to determine the evolutionary trajectories, the sequences containing the different mutations were amplified and Sanger-sequenced from stored samples of the four populations at days 5, 10, 15, 20, 25 and 30 (Table 1). Tigecycline susceptibility was also evaluated at these time points. Figure 3 shows the order of appearance of the genetic changes, as well as the increase in the tigecycline MICs, along the 30-days evolutions. Although every population has reached resistance to tigecycline through the accumulation of different mutations, some common aspects can be found. The first mutation selected in all the populations is located in the SmeDEF regulator *smeT*, leading to a L166Q substitution in populations A, B and D, and a T197P change in population C. Together with previously published epidemiological work, this result suggests that *smeDEF* overexpression should contribute to the acquisition of resistance by *S. maltophilia.* [24] To analyze this possibility, the tigecycline susceptibility of the wild-type D457 strain, and that of the SmeDEF overproducer strain D57R, [29] was analyzed. Tigecycline MIC raised from 1 mg/L in the wild-type strain to 3 mg/L in D457R, indicating that *smeDEF* overexpression contributes to *S. maltophilia* tigecycline resistance.

Mutations in *frr* were detected in the four populations between days 10 and 15 of evolution, while the 300-kbp duplication-inversion of population C was detected at day 20 of evolution. In other cases, such as *SMD_RS15850* or *dedD*, the low frequency of the mutations did not allow to detect the genetic change within the whole population (Table 1).

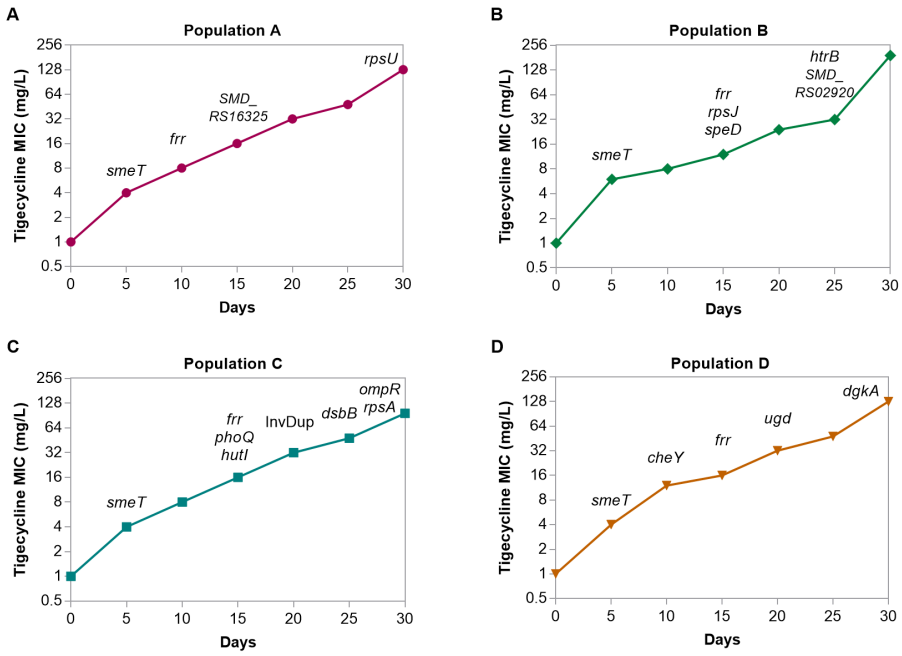

Figure 3. Mutational trajectories and tigecycline resistance during the 30-days tigecycline experimental evolution. The MICs of tigecycline and the order of appearance of the genetic changes was determined in populations A (A), B (B), C (C) and D (D) from samples stored during the evolution period every 5 days by PCR amplification and sequencing. Those dots without any gene name indicate that no new mutations were detected in the corresponding day. MIC, minimum inhibitory concentration; InvDup, inverted duplication.

Antibiotic susceptibility profile of the tigecycline-evolved populations

To analyze the influence of the mutations selected under tigecycline pressure on the susceptibility to other antimicrobials, the MICs of antibiotics belonging to different families were determined at the end of evolution (Supplementary Table S3). All the evolved populations present a decreased susceptibility to quinolones, chloramphenicol, streptomycin and tetracycline (Figure 4), suggesting that not all the selected mutations are tigecycline-specific. A low susceptibility for aztreonam, erythromycin and gentamicin is also observed for some of the populations. Regarding collateral sensitivity, the four evolved populations are more susceptible to fosfomycin. In addition, population C presents collateral susceptibility to SXT. All populations present mutations in the *smeDEF* repressor *smeT*. Since *smeDEF* overexpression is associated with a reduced susceptibility to SXT in *S. maltophilia*, [14] it might be possible that the collateral susceptibility of population C to SXT could be due to mutations selected after the first selection of the *smeT* mutation. To address this possibility, the susceptibility to SXT was measured in all the populations at day 5 of evolution. In all cases, the populations present a decreased susceptibility to SXT, consistent with the presence of *smeT* mutants in these populations (not shown). Finally, population D shows collateral sensitivity to aztreonam, despite the low susceptibility observed to this antibiotic in the other populations.

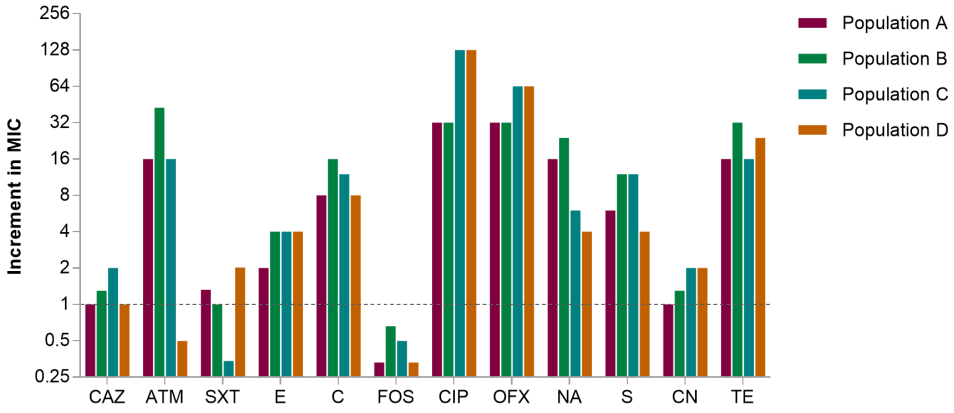

Figure 4. Susceptibility of the *S. maltophilia* evolved populations to antibiotics from different families. MICs were determined in the four tigecycline-evolved populations from the stored samples of day 30 of evolution. MICs changes were determined using the MIC values of the wild-type strain D457 as reference (dashed line). MIC, minimum inhibitory concentration; CAZ, ceftazidime; ATM, aztreonam; SXT, trimethoprim/sulfamethoxazole; E, erythromycin; C, chloramphenicol; FOS, fosfomycin; CIP, ciprofloxacin; OFX, ofloxacin; NA, nalidixic acid; S, streptomycin; CN, gentamicin; TE, tetracycline.

Fitness cost associated with tigecycline resistance during experimental evolution

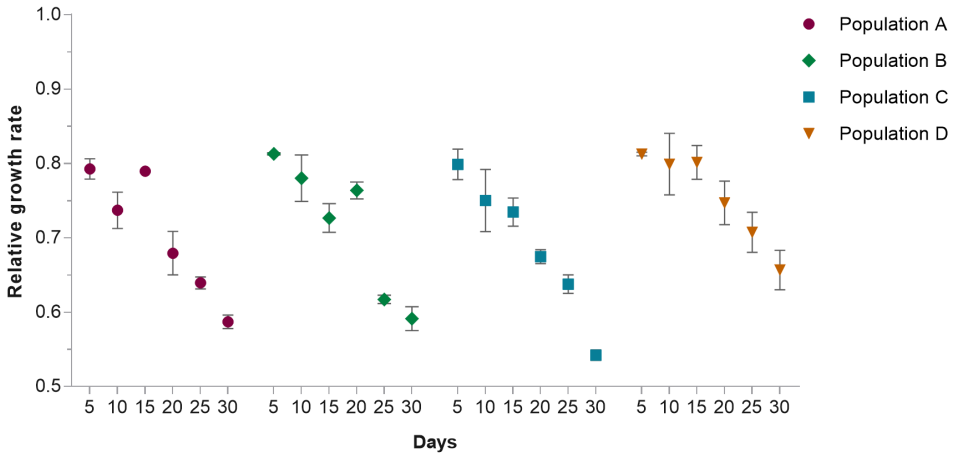

Figure 5. Fitness costs of the tigecycline-evolved *S. maltophilia* populations. Growth experiments were performed in the four evolved populations from samples stored during the evolution period every 5 days. Growth rates were calculated from OD_{600} values corresponding to exponential growth. Relative growth rates were calculated using the wild-type D457 value as a reference (1). Error bars represent the standard deviation from three independent replicates.

The relative fitness costs of the four tigecycline-evolved populations were assessed at days 5, 10, 15, 20, 25 and 30 of evolution by measuring their growth rates and comparing with that of the parental strain D457. All the populations show around a 20 % deficiency in their growth rate at day 5 of evolution (Figure 5). During the following days, while tigecycline concentration increases, mutations accumulate in the bacteria and fitness costs becomes notably higher, ranging from 35 % to 45 % at the end of the experiment. Interestingly, in some cases the fitness cost seems to be recovered at different days. For instance, in population A, the relative fitness cost is 27 % at day 10; however, at day 15, it is reduced to 21 %, suggesting that compensatory mutations are selected.

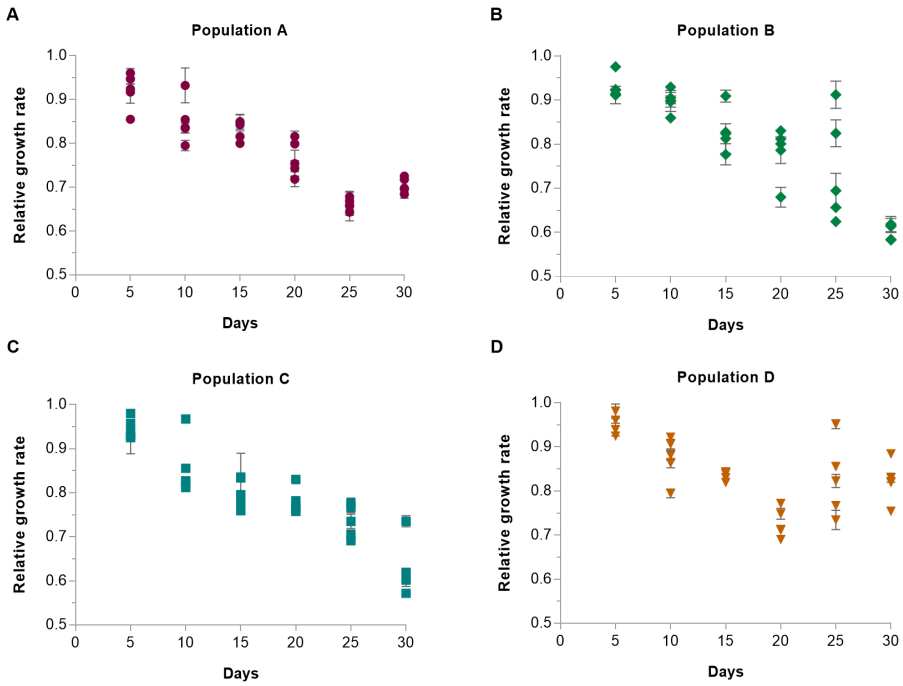

Figure 6. Fitness costs of isolated clones from the tigecycline-evolved *S. maltophilia* populations. Growth experiments were performed in five single clones isolated from the four evolved populations from samples stored during the evolution period every 5 days. Growth rates were calculated from OD_{600} values corresponding to exponential growth. Relative growth rates were calculated using the wild-type D457 value as a reference (1). Error bars represent the standard deviation from three independent replicates.

With the aim of determining whether the tigecycline resistance-associated fitness costs of the evolved populations were also observed in individual clones, five single colonies from each population and time points (5, 10, 15, 20, 25 and 30) were isolated on LB agar plates and the relative fitness of each clone was assessed. As shown in Figure 6, all the isolated colonies present a defect in growth in comparison with the wild-type strain from day 5 of evolution, although, in some cases, these costs are not as accused as the ones observed in the population setting. In the case of the colonies isolated from days 25 from the populations B and D, some of them present a better growth rate in comparison with the previous time point, indicating possible compensatory events during the evolution.

X

Discussion

Using *in vitro* experimental evolution, we have determined the adaptive changes and the phenotypical consequences driven by the exposure of *S. maltophilia* to increasing concentrations of tigecycline. The tigecycline-evolved populations reached high-level resistance to this antibiotic in a step-wise manner through the accumulation of sequential mutations in different genetic elements. The evolutionary trajectories followed by the four parallel cultures show that the first event in the evolution leading to tigecycline resistance is a mutation in *smeT*. Mutations in this regulator have been previously associated with antibiotic resistance in *S. maltophilia* clinical isolates. Indeed, both L166Q and T197P amino acid substitutions have been already found in *S. maltophilia* clinical isolates that overexpress *smeDEF*. [29,30] Further, L166Q in SmeT has been detected more recently in a clinical isolate from a patient with bacteremia treated with the quinolone levofloxacin. [31] These findings support that the results derived from our *in vitro* analysis can be translated to the clinical settings. With the exception of *smeT* mutations, which have been already detected in *S. maltophilia* clinical isolates, none of the other mutations were found in the available genomes of *S. maltophilia* strains after an *in silico* analysis in GenBank, but some of them have been associated with resistance, or have been found in clinical isolates, in other bacterial pathogens (see below).

The second common aspect among the four evolutions is the selection of two different SNPs in the ribosome recycling factor *frr*. A different mutation that those obtained in this work has been previously found in *frr* after a similar 35-days experimental evolution in the presence of tigecycline in *Pseudomonas aeruginosa*, [32] confirming that modifications in the ribosome result in tigecycline resistance. Other ribosome-related genes selected in this study have been previously reported to cause antibiotic resistance in other bacterial species. For instance, *rpsJ*, which encodes for the 30S ribosomal protein S10, has been described as a general marker of adaptation and reduced susceptibility to tigecycline, [33] since mutations in this gene have been detected after experimental evolutions in the presence of this antibiotic in different bacterial pathogens, [32-34] as well as in tigecycline-resistant *K. pneumoniae* clinical isolates. [35] Furthermore, an V57L amino acid substitution in RpsJ has been recently detected in a *K. pneumoniae* clinical isolate from a patient undergoing tigecycline therapy, indicating that tigecycline resistance can occur *in vivo* through the selection of mutations in the ribosomal S10-coding gene. [36] Mutations in the genes encoding the 30S ribosomal proteins S21 (*rpsU*) and S1 (*rpsA*) have also been detected. In contrast to *frr* and *rpsJ*, this is the first time that *rpsU* and *rpsA* are identified to have a role in tigecycline resistance.

Tigecycline exposure has also driven to the selection of mutations in different *S. maltophilia* genes involved in the LPS biosynthesis and membrane homeostasis. LPS is a major component of the outer membrane in Gram-negatives, being involved in pathogen-host interactions during infections, antimicrobials resistance and virulence. [37-39] In our study, amino acid substitutions were found in the lipid A biosynthetic enzymes lauroyl acyltransferase (*htrB*) and phosphoethanolamine transferase (*SMD_RS16325)*. HtrB performs one of the last modifications that lipid A suffers before being transported to the outer membrane. [40] Regarding antibiotic resistance, we found two amino acid changes (V236I and D246N) in the HtrB protein of a

tigecycline-resistant *K. pneumoniae* clinical isolate from a patient with bacteraemia through a search in the Pathosystems Resource Integration Center (PATRIC) database. [41] Nevertheless, the particular contribution of these mutations to the tigecycline resistance phenotype is unknown. Besides, mutants of *P. aeruginosa* lacking two *htrB* homologs display a phenotype of increased susceptibility to antimicrobial peptides and cell wall inhibitor antibiotics. [42] SMD_RS16325, a homolog of the *E. coli* phosphoethanolamine transferase EptA, modifies the 1 and 4' headgroups of the lipid A with phosphoethanolamine. [43] Mutants of *Neisseria gonorrhoeae* lacking its EptA homolog (LptA) show an increased susceptibility to antimicrobial peptides and an attenuated colonization of mice genital tract and human urethra. [44] Other LPS-related genes that were found mutated after the tigecycline challenge were *ugd*, encoding an UDP-glucose dehydrogenase that converts UDP-glucose into the UDP-glucuronic acid necessary for the synthesis of extracellular polysaccharides and LPS in many pathogenic bacteria; [45,46] and the two-component sensor histidine kinase *phoQ*, reported to be involved in polymyxin resistance through lipid A modification in *P. aeruginosa* isolates from cystic fibrosis patients. [47] A 6-bp insertion was also detected in the diacylglycerol kinase-coding gene (*dgkA*), which is involved in the synthesis of phosphatidic acid, the universal precursor of phospholipids. [48] Although the mechanism of tigecycline uptake by the cell is not known, it is generally assumed that it follows the same pathway as tetracycline, either by diffusing through the outer membrane lipid barrier or via porins. [49,50] It is possible that mutations occurring in LPS-related genes lead to modifications of the bacterial outer membrane, preventing the uptake of tigecycline, assuming that it diffuses through the membrane, and explaining the observed increment of resistance against this antibiotic. [23]

Genetic modifications were also found in other *S. maltophilia* enzymes. For instance, a V117A change was detected in *speD*, which encodes a S-adenosylmethionine decarboxylase involved in the synthesis of polyamines. [51] Polyamines are polycationic compounds that modulate gene expression leading to optimal cell growth and defense against adverse conditions. [52,53] Mutations in *speD* have been linked to high-level resistance against the aminoglycoside kasugamycin in *Bacillus subtilis*. [54] Also, a A20V change was detected in the *dsbB* gene. The DsbA/DsbB system mediates the disulfide bonds formation, required for protein stability, activity and folding of a wide range of proteins, including those involved in virulence. [55] *dsbB* mutants present an attenuated virulence and an increase sensitivity to a variety of antibiotics in other bacterial species. [56,57] Although the molecular mechanisms by which these mutations lead to tigecycline resistance in *S. maltophilia* remain to be clarified, our data highlight the variety of resources that this bacterium has to overcome tigecycline exposure.

A fact to be highlighted from this work is the presence of two inverted duplications comprising 262 and 53 genes, respectively (Supplementary Table S2). A wide variety of genes, including transporters, two-component systems, transcriptional regulators and enzymes, are included in these regions and might potentially contribute to tigecycline resistance. Genome rearrangements, such as deletions, duplications or large chromosomal inversions, as the ones present in our study, are responsible of chromosomal stability and variation, which can be relevant for niche occupation and/or adaptation in pathogenic and non-pathogenic bacteria. [58,59] Besides, gene inversion can lead to an increased bacterial virulence and drug resistance,

promoting as well bacterial evolvability. [60] Notwithstanding the fact that these chromosomal rearrangements arise frequently, they are usually lost due to its instability. [61] However, in our experiment, the 300-kbp duplication-inversion is first detected at day 20 and maintained until the end of the evolution, suggesting that it confers a selective advantage to bacteria growing in the presence of tigecycline. Further, the 53 genes from the 60-kpb duplicated-inverted region in population D are also included in the 300-kbp sequence from population C, suggesting that in this region there might be genes whose duplication and/or inversion causes an increment in tigecycline resistance or a fitness cost compensation.

The acquisition of mutations in relevant resistance determinants may imply that the susceptibility to other antibiotics might be affected as well. To test this possibility, the susceptibility to other antibiotics belonging to different families was assessed in all the tigecycline-evolved populations. The observed cross-resistance to quinolones, chloramphenicol, erythromycin and tetracycline in all the populations is consistent with the fact that these antimicrobials are substrates of SmeDEF, which would be overexpressed because of *smeT* mutation. [62] Acquisition of tetracycline resistance can also be explained by the selected mutations in some ribosome-related genes, which might be also a target of this antibiotic, [63] as well as of streptomycin. [64] Remarkably, all the evolved populations show an increased susceptibility to fosfomycin in comparison with the parental strain. This is in agreement with the study of Sanz-García *et al*., [32] where exposure to tigecycline renders high susceptibility to fosfomycin in *P. aeruginosa*. Besides these common features, we also found some differences in the susceptibilities to other antibiotics. For instance, populations C and D displayed a higher susceptibility to SXT and aztreonam, respectively, despite the fact that populations A, B and C showed an increased resistance to the last one. These results suggest that the different mutational trajectories render different outcomes regarding susceptibility to other antibiotics, not used in selection.

One of the key factors that influences the emergence and persistence of antibiotic resistant bacteria is the resistance-associated fitness cost. The acquisition of resistance entails a fitness advantage in the presence of antibiotics in comparison with its susceptible counterpart. Nevertheless, in the absence of selective pressure, antibiotic resistance may lead to a fitness burden, thus resistant bacteria would be outcompeted by the susceptible ones. [65,66] Our data show that, in the absence of tigecycline, the four evolved populations, as well as the single isolated clones, present a considerable fitness cost. Although we have highlighted the role of several of these mutations in antimicrobial resistance, it is possible that some of them were selected in order to compensate these costs. These results suggest that, in the absence of antibiotics, it could be difficult for these mutants to be maintained in the overall susceptible population. Nevertheless, they will re-emerge when *S. maltophilia* is confronted to antibiotic selective pressure, being an example of short-sighted evolution. [67]

The use of tigecycline in combination with quinolones have been proposed for treating *S. maltophilia* infections. [68] Our findings that all evolved populations present increased quinolones MICs introduces some concerns on this possibility. However, tigecycline/fosfomycin combinations might be taken into consideration since all tigecycline-resistant populations present fosfomycin collateral susceptibility. Finally, the fact that *smeDEF* overexpression stands as a major element in quinolones, SXT and tigecycline

resistance, which are the most useful antibiotics against *S. maltophilia*, is of special concern since, once a *smeDEF*-overexpressing mutant is selected by the treatment with any of these antibiotics, it will be resistant to all of them.

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Transparency declarations

None to declare

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Mechanisms and Phenotypic Consequences of Tigecycline Resistance Acquisition in *Stenotrophomonas maltophilia*

Supplementary Material

Table S1. Primers used in this study

Name	Sequence (5'-3')	Utilization
smeT_L	GAGGGAGCTGATATCGATGG	Verification of L166Q and T197P in *smeT*
smeT_R	GATGGGAGCAGCCTCGTT	
frr_L	CGTCCCGTATTCGCCTATAA	Verification of T50P and P90Q in *frr*
frr_R	GAAGGCATCGACTTCAGACC	
smd_3146_L	CTACATGAGCCGCTACCACA	Verification of S284L in *SMD_RS16325*
smd_3146_R	CAGGATCTCGTCCATGCAG	
rpsU_L	TCGCATGCCTGCTATTATGA	Verification of E24L in *rpsU*
rpsU_R	GAACCTCGTGGTGGTAGGTG	
rpsJ_L	AGGTCCATGGAGTGACCTTG	Verification of V73G in *rpsJ*
rpsJ_R	GGCCATCTTCGGTGAACA	
htrB_L	GTAGAATGGCCCCATGTCAG	Verification of H290R in *htrB*
htrB_R	CACCGCATCCGATTATTTGT	
speD_L	GCCAGAACTCTTGCGTCTTC	Verification of V117A in *speD*
speD_R	GGCTTACTCGGACAGGTTCC	
smd_0534_L	TTTTGGCACGTTGGTGAGTA	Verification of Gln364* in *SMD_RS02920*
smd_0534_R	AGGTTGGAGATGGACTGTGG	
hutI_L	GAGGAATGCCGGGTCAAC	Verification of G283D in *hutI*
hutI_R	TGGAGCAACACATGAGCAA	

phoQ_tig3_L	CGAATTCCCGTACACCATCT	Verification of V279L in *phoQ*
phoQ_tig3_R	CACTTGAAGGCGTTCTCCAG	
dsbB_2_L	GCTCTCGGTTACGCTTGG	Verification of A20V in *dsbB*
dsbB_2_R	GACCCCATCCTGCTTCA	
smd_3368_L	CCTGTATCGTTCAGCAGGTA	Verification of L54fs in *ompR*
smd_3368_R	ATCAGCGGCACTTCAAAC	
rpsA_L	GTTCTCGCTGACCGTGCT	Verification of N463fs in *rpsA*
rpsA_R	GTGAACAACATTGCCGACAG	
cheY_L	TGGTACGAACTTCCGTGGAT	Verification of E21K in *cheY*
cheY_R	CGACACTGAATACTGCAATCG	
smd_2759_L	CCTGCCGTAAGCGACCAC	Verification of F66S in *ugd*
smd_2759_R	ATCTTGGTCGCCAGCATC	
dgkA_L	AAGGGGAGCTCCATCATAG	Verification of L71_A72insVL in *dgkA*
dgkA_R	ATGGAAATGAACACCAGGTT	
TigC_1_L	GGTGGTCCTTCCTGCTTG	Verification of the inverted duplication in lineage C
TigC_2_L	CCAGGAGCAGATCGAGATT	

Table S2. Genes included in the inverted duplications of the evolved populations C and D

Chromosome region		Gene	Locus tag	Protein name	Population
1330809	1333607	-	SMD_RS06300	TonB-dependent receptor	C
1333622	1334590	-	SMD_RS06305	DUF4880 domain-containing protein	C
1334587	1335120	-	SMD_RS06310	sigma-70 family RNA polymerase sigma factor	C
1335353	1338226	-	SMD_RS06315	autotransporter domain-containing protein	C
1338324	1339325	-	SMD_RS06320	2-hydroxyacid dehydrogenase	C
1339547	1344934	-	SMD_RS22010	RHS repeat protein	C
1344903	1345871	-	SMD_RS22260	hypothetical protein	C
1345875	1346489	-	SMD_RS22265	DUF416 family protein	C
1346568	1346978	-	SMD_RS22775	hypothetical protein	C
1346989	1347957	-	SMD_RS22270	hypothetical protein	C
1347954	1348280	-	SMD_RS06335	hypothetical protein	C
1348535	1348870	-	SMD_RS06345	hypothetical protein	C
1348884	1349519	-	SMD_RS06350	hypothetical protein	C
1349875	1350915	*cyoA*	SMD_RS06355	MULTISPECIES: ubiquinol oxidase subunit II	C
1350918	1352915	*cyoB*	SMD_RS06360	cytochrome o ubiquinol oxidase subunit I	C
1352912	1353550	*cyoC*	SMD_RS06365	cytochrome o ubiquinol oxidase subunit III	C
1353550	1353891	*cyoD*	SMD_RS06370	MULTISPECIES: cytochrome o ubiquinol oxidase subunit IV	C
1354249	1356306	-	SMD_RS06375	bifunctional diguanylate cyclase/phosphodiesterase	C
1356456	1357832	-	SMD_RS06380	MULTISPECIES: DNA repair protein RadA	C
1357968	1359827	-	SMD_RS06385	histidine kinase	C
1359876	1362182	-	SMD_RS06390	hypothetical protein	C
1362290	1362754	-	SMD_RS06395	hypothetical protein	C
1362854	1363648	-	SMD_RS06400	membrane protein	C

1363771	1365147	-	SMD_RS06405	MULTISPECIES: signal recognition particle protein	C
1365296	1366372	-	SMD_RS06410	nitronate monooxygenase	C
1366369	1367181	-	SMD_RS06415	aminotransferase	C
1367293	1367553	-	SMD_RS06420	MULTISPECIES: 30S ribosomal protein S16	C
1367598	1368110	-	SMD_RS06425	MULTISPECIES: ribosome maturation factor RimM	C
1368119	1368877	-	SMD_RS06430	tRNA (guanosine(37)-N1)-methyltransferase TrmD	C
1369032	1369433	-	SMD_RS06435	MULTISPECIES: 50S ribosomal protein L19	C
1369516	1369896	-	SMD_RS06440	hypothetical protein	C
1369976	1370410	-	SMD_RS06445	DUF1801 domain-containing protein	C
1370449	1370856	-	SMD_RS06450	DUF1801 domain-containing protein	C
1370974	1372434	-	SMD_RS06455	MATE family efflux transporter	C
1372497	1372736	-	SMD_RS06460	hypothetical protein	C
1372746	1373153	-	SMD_RS06465	RNA-binding S4 domain-containing protein	C
1373192	1373794	-	SMD_RS06470	DUF937 domain-containing protein	C
1373903	1375546	-	SMD_RS06475	catalase	C
1375736	1378327	*mutS*	SMD_RS06480	DNA mismatch repair protein MutS	C
1378463	1378855	-	SMD_RS06485	hypothetical protein	C
1379185	1379376	-	SMD_RS06490	hypothetical protein	C
1379659	1379850	-	SMD_RS06495	hypothetical protein	C
1379849	1380286	-	SMD_RS06500	hypothetical protein	C
1380482	1380802	-	SMD_RS06505	hypothetical protein	C
1380803	1380985	-	SMD_RS22275	DUF596 domain-containing protein	C
1381276	1383498	-	SMD_RS06510	methyl-accepting chemotaxis protein	C
1383665	1384033	-	SMD_RS06515	MULTISPECIES: glycine zipper 2TM domain-containing protein	C
1384162	1385289	-	SMD_RS06520	ABC transporter permease	C
1385286	1386473	-	SMD_RS06525	ABC transporter permease	C

1386458	1387471	-	SMD_RS06530	HlyD family efflux transporter periplasmic adaptor subunit	C
1387464	1388864	-	SMD_RS06535	TolC family protein	C
1388943	1389836	-	SMD_RS06540	LysR family transcriptional regulator ArgP	C
1389943	1390557	-	SMD_RS06545	amino acid transporter	C
1390554	1391342	*map*	SMD_RS06550	type I methionyl aminopeptidase	C
1391344	1391556	-	SMD_RS06555	hypothetical protein	C
1391625	1391984	-	SMD_RS06560	VOC family protein	C
1392124	1393008	-	SMD_RS06565	hypothetical protein	C
1393012	1394076	-	SMD_RS06570	GNAT family N-acetyltransferase	C
1394105	1395505	-	SMD_RS06575	aminotransferase class III-fold pyridoxal phosphate-dependent enzyme	C
1395570	1396286	-	SMD_RS06580	phosphatase	C
1396455	1397663	-	SMD_RS06585	nucleotide sugar aminotransferase	C
1397674	1398072	-	SMD_RS06590	EamA family transporter	C
1398069	1398428	-	SMD_RS06595	MULTISPECIES: membrane protein	C
1398430	1399266	-	SMD_RS06600	alpha/beta fold hydrolase	C
1399347	1400726	-	SMD_RS06605	sensor histidine kinase	C
1400723	1401406	-	SMD_RS06610	response regulator	C
1401540	1402667	-	SMD_RS06615	metallophosphoesterase	C
1402741	1403190	-	SMD_RS06620	transcriptional regulator	C
1403205	1404467	-	SMD_RS06625	MFS transporter	C
1404563	1405978	-	SMD_RS06630	diguanylate cyclase	C
1406265	1408514	-	SMD_RS06635	TonB-dependent siderophore receptor	C
1408698	1409678	-	SMD_RS06640	cation transporter	C
1409717	1410391	-	SMD_RS06645	deoxyribonuclease V	C
1410485	1411234	*gpmA*	SMD_RS06650	MULTISPECIES: 2,3-diphosphoglycerate-dependent phosphoglycerate mutase	C

1411345	1411812	-	SMD_RS06655	hypothetical protein	C
1411944	1414037	-	SMD_RS06660	M13 family peptidase	C
1414238	1414855	-	SMD_RS06665	HD domain-containing protein	C
1414901	1418365	-	SMD_RS06670	transcription-repair coupling factor	C
1418515	1419060	-	SMD_RS06675	MULTISPECIES: GNAT family N-acetyltransferase	C
1419156	1420013	-	SMD_RS06680	23S rRNA (adenine(2030)-N(6))-methyltransferase RlmJ	C
1420049	1420774	-	SMD_RS06685	two-component system response regulator CreB	C
1420778	1422238	-	SMD_RS06690	two-component system sensor histidine kinase CreC	C
1422320	1423639	-	SMD_RS06695	cell envelope integrity protein CreD	C
1423650	1424060	-	SMD_RS06700	hypothetical protein	C
1424216	1425808	-	SMD_RS06705	M20/M25/M40 family metallo-hydrolase	C and D
1425963	1427366	-	SMD_RS06710	glutamate--tRNA ligase	C and D
1427401	1427892	-	SMD_RS06715	MULTISPECIES: Fur family transcriptional regulator	C and D
1428004	1429158	-	SMD_RS06720	HlyD family secretion protein	C and D
1429167	1430840	-	SMD_RS06725	MFS transporter	C and D
1430840	1431406	-	SMD_RS06730	TetR/AcrR family transcriptional regulator	C and D
1431606	1433816	-	SMD_RS06735	TonB-dependent receptor	C and D
1433953	1434366	-	SMD_RS06740	MULTISPECIES: MerC domain-containing protein	C and D
1434477	1434656	-	SMD_RS06745	MULTISPECIES: 30S ribosomal protein THX	C and D
1434787	1436037	-	SMD_RS06750	alkaline phosphatase family protein	C and D
1436142	1436633	-	SMD_RS06755	methylated-DNA-	C and D
1436630	1438108	-	SMD_RS06760	DNA-3-methyladenine glycosylase 2 family protein	C and D
1438579	1439742	-	SMD_RS06765	hypothetical protein	C and D
1439801	1440346	-	SMD_RS06770	DUF4019 domain-containing protein	C and D
1440358	1440747	-	SMD_RS06775	membrane protein	C and D
1440827	1442005	-	SMD_RS06780	FAD-dependent hydroxylase	C and D

1442017	1442238	-	SMD_RS06785	MULTISPECIES: RNA-binding S4 domain-containing protein	C and D
1442505	1444442	-	SMD_RS06790	DEAD/DEAH box helicase	C and D
1444661	1447084	-	SMD_RS06795	EAL domain-containing protein	C and D
1447194	1448858	-	SMD_RS06800	diguanylate cyclase	C and D
1448893	1449615	-	SMD_RS06805	pseudouridine synthase	C and D
1449733	1450803	-	SMD_RS06810	sensor domain-containing diguanylate cyclase	C and D
1450946	1452106	-	SMD_RS06815	ATP-binding protein	C and D
1452137	1453084	-	SMD_RS06820	DMT family transporter	C and D
1453164	1453778	-	SMD_RS06825	hypothetical protein	C and D
1453699	1454574	-	SMD_RS06830	DUF72 domain-containing protein	C and D
1454571	1455014	-	SMD_RS06835	glyoxalase	C and D
1455125	1456687	-	SMD_RS06840	serine hydrolase	C and D
1456844	1458676	-	SMD_RS06845	multidrug efflux ABC transporter SmrA	C and D
1458735	1459859	-	SMD_RS06850	hypothetical protein	C and D
1459793	1460770	-	SMD_RS06855	hypothetical protein	C and D
1462063	1463331	-	SMD_RS06860	bifunctional glucose-1-phosphatase/inositol phosphatase	C and D
1463341	1464339	-	SMD_RS06865	polysaccharide lyase	C and D
1464551	1464787	-	SMD_RS06870	hypothetical protein	C and D
1464877	1466685	-	SMD_RS06875	DUF885 domain-containing protein	C and D
1466798	1467154	-	SMD_RS06880	GFA family protein	C and D
1467151	1467966	-	SMD_RS06885	DUF4349 domain-containing protein	C and D
1467998	1468276	-	SMD_RS06890	hypothetical protein	C and D
1468360	1469304	-	SMD_RS06895	LysR family transcriptional regulator	C and D
1469420	1470292	-	SMD_RS06900	DMT family transporter	C and D
1470881	1471783	-	SMD_RS06905	peptidylprolyl isomerase	C and D
1471920	1472285	-	SMD_RS06910	MULTISPECIES: PadR family transcriptional regulator	C and D

1472282	1473271	-	SMD_RS06915	membrane protein	C and D
1473382	1474689	-	SMD_RS06920	polyhydroxyalkanoate depolymerase	C and D
1474783	1475373	-	SMD_RS06925	class I SAM-dependent methyltransferase	C and D
1475523	1475978	-	SMD_RS06930	membrane protein	C and D
1476118	1477077	-	SMD_RS06935	acetyl-CoA carboxylase carboxyl transferase subunit alpha	C and D
1477191	1478060	-	SMD_RS06940	hypothetical protein	C and D
1478125	1481715	-	SMD_RS06945	DNA polymerase III subunit alpha	C and D
1481953	1482612	-	SMD_RS06950	ribonuclease HII	C and D
1482609	1483868	-	SMD_RS06955	MULTISPECIES: lipid-A-disaccharide synthase	C and D
1483865	1484656	-	SMD_RS06960	acyl-ACP--UDP-N-acetylglucosamine O-acyltransferase	C and D
1484673	1485131	*fabZ*	SMD_RS06965	MULTISPECIES: 3-hydroxyacyl-ACP dehydratase FabZ	C and D
1485128	1486150	*lpxD*	SMD_RS06970	UDP-3-O-(3-hydroxymyristoyl) glucosamine N-acyltransferase	C
1486533	1488896	*bamA*	SMD_RS06975	outer membrane protein assembly factor BamA	C
1488971	1490329	*rseP*	SMD_RS06980	RIP metalloprotease RseP	C
1490385	1491575	-	SMD_RS06985	1-deoxy-D-xylulose-5-phosphate reductoisomerase	C
1491578	1492414	-	SMD_RS06990	hypothetical protein	C
1492411	1493178	*uppS*	SMD_RS06995	MULTISPECIES: di-trans,poly-cis-decaprenylcistransferase	C
1493184	1493738	-	SMD_RS07000	MULTISPECIES: ribosome recycling factor	C
1493835	1494563	-	SMD_RS07005	MULTISPECIES: UMP kinase	C
1494685	1496223	-	SMD_RS07010	GGDEF domain-containing protein	C
1496481	1497356	*tsf*	SMD_RS07015	elongation factor Ts	C
1497482	1498288	*rpsB*	SMD_RS07020	MULTISPECIES: 30S ribosomal protein S2	C
1498509	1499207	-	SMD_RS07025	pilus assembly protein	C
1499207	1499422	-	SMD_RS07030	hypothetical protein	C
1499575	1500612	-	SMD_RS07035	spore coat U domain-containing protein	C
1500609	1502978	-	SMD_RS07040	fimbrial biogenesis outer membrane usher protein	C

1502986	1503735	-	SMD_RS07045	molecular chaperone	C
1503735	1504265	-	SMD_RS07050	SCPU domain-containing protein	C
1504439	1505281	*map*	SMD_RS07055	type I methionyl aminopeptidase	C
1505284	1507911	*glnD*	SMD_RS07060	[protein-PII] uridylyltransferase [Stenotrophomonas maltophilia]	C
1507911	1508990	*dapD*	SMD_RS07065	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase	C
1508993	1509859	-	SMD_RS07070	hypothetical protein	C
1509897	1510262	-	SMD_RS07075	arsenate reductase	C
1510259	1511386	*dapE*	SMD_RS07080	succinyl-diaminopimelate desuccinylase	C
1511507	1512385	-	SMD_RS07085	sel1 repeat family protein	C
1512724	1514415	*asnB*	SMD_RS07090	asparagine synthase B	C
1514517	1515905	-	SMD_RS07095	sodium:proton symporter	C
1515988	1518315	-	SMD_RS07100	penicillin acylase family protein	C
1518439	1518927	*bfr*	SMD_RS07105	MULTISPECIES: bacterioferritin	C
1519320	1521563	*parC*	SMD_RS07110	DNA topoisomerase IV subunit A	C
1521624	1522532	-	SMD_RS07115	AraC family transcriptional regulator	C
1522678	1523121	-	SMD_RS07120	MULTISPECIES: multidrug efflux system transcriptional regulator EmrR	C
1523118	1524611	-	SMD_RS07125	multidrug efflux transporter outer membrane subunit EmrC	C
1524623	1525804	-	SMD_RS07130	multidrug efflux MFS transporter periplasmic adaptor subunit EmrA	C
1525812	1527398	*emrB*	SMD_RS07135	MULTISPECIES: multidrug efflux MFS transporter permease subunit EmrB	C
1527687	1528586	-	SMD_RS07145	LysR family transcriptional regulator	C
1528685	1529431	-	SMD_RS07150	sulfite exporter TauE/SafE family protein	C
1529993	1532452	*rnr*	SMD_RS07165	ribonuclease R	C
1532537	1533019	-	SMD_RS07170	GFA family protein	C
1533181	1533924	-	SMD_RS07175	MULTISPECIES: 23S rRNA (guanosine(2251)-2\'-O)-methyltransferase RlmB	C
1534002	1535441	-	SMD_RS07180	TolC family protein	C

1535438	1537396	-	SMD_RS07185	MacB family efflux pump subunit	C
1537393	1538634	-	SMD_RS07190	efflux RND transporter periplasmic adaptor subunit	C
1538839	1539567	-	SMD_RS07195	response regulator	C
1541161	1544187	-	SMD_RS07200	diguanylate cyclase	C
1544350	1545000	*rnt*	SMD_RS07205	ribonuclease T	C
1545132	1545653	-	SMD_RS07210	DoxX family protein	C
1545678	1546433	-	SMD_RS07215	hypothetical protein	C
1546426	1547301	-	SMD_RS07220	DUF692 domain-containing protein	C
1547288	1547641	-	SMD_RS07225	hypothetical protein	C
1547878	1548585	*phoU*	SMD_RS07230	MULTISPECIES: phosphate signaling complex protein PhoU	C
1548662	1549492	-	SMD_RS07235	MULTISPECIES: phosphate ABC transporter ATP-binding protein PstB	C
1549512	1550375	*pstA*	SMD_RS07240	phosphate ABC transporter permease PstA	C
1550375	1551343	*pstC*	SMD_RS07245	MULTISPECIES: phosphate ABC transporter permease subunit PstC	C
1551423	1552511	*pstS*	SMD_RS07250	phosphate ABC transporter substrate-binding protein PstS	C
1552928	1553944	*pstS*	SMD_RS07255	MULTISPECIES: phosphate ABC transporter substrate-binding protein PstS	C
1554499	1555731	-	SMD_RS07260	porin	C
1555813	1556505	*nth*	SMD_RS07265	endonuclease III	C
1556505	1556858	-	SMD_RS07270	hypothetical protein	C
1556863	1557645	-	SMD_RS07275	enoyl-CoA hydratase	C
1557813	1558514	-	SMD_RS07280	FKBP-type peptidyl-prolyl cis-trans isomerase	C
1558681	1559484	-	SMD_RS07285	CoA pyrophosphatase	C
1559481	1560383	-	SMD_RS07290	sulfurtransferase	C
1560646	1561212	-	SMD_RS07295	N-acetylmuramoyl-L-alanine amidase	C
1561285	1561812	-	SMD_RS07300	alpha/beta hydrolase	C
1561958	1564096	-	SMD_RS07305	bifunctional 23S rRNA (guanine(2069)-N(7))-methyltransferase RlmK/23S rRNA (guanine(2445)-N(2))-methyltransferase RlmL	C

1564217	1564546	-	SMD_RS07310	DUF3325 domain-containing protein	C
1564543	1566180	-	SMD_RS07315	PepSY domain-containing protein	C
1566177	1566491	-	SMD_RS07320	DUF3649 domain-containing protein	C
1566737	1568023	-	SMD_RS07325	DUF445 family protein	C
1568148	1569026	-	SMD_RS07330	hypothetical protein	C
1569090	1570442	-	SMD_RS07335	aspartate aminotransferase family protein	C
1570546	1572042	-	SMD_RS07340	aldehyde dehydrogenase	C
1572243	1573544	-	SMD_RS07345	MFS transporter	C
1573525	1573986	-	SMD_RS07350	MULTISPECIES: hemerythrin	C
1574072	1575415	-	SMD_RS07355	diguanylate cyclase	C
1575512	1577074	-	SMD_RS07360	membrane protein	C
1577534	1578022	-	SMD_RS07365	hypothetical protein	C
1578181	1579773	-	SMD_RS07370	membrane protein	C
1579999	1581276	-	SMD_RS07375	FAD-binding oxidoreductase	C
1581438	1582199	-	SMD_RS07380	gamma-glutamyl-gamma-aminobutyrate hydrolase	C
1582196	1583593	-	SMD_RS07385	glutamine synthetase	C
1583686	1584795	-	SMD_RS07390	polyamine ABC transporter substrate-binding protein	C
1584985	1586121	*potG*	SMD_RS07395	polyamine ABC transporter ATP-binding protein	C
1586118	1587050	-	SMD_RS07400	putrescine ABC transporter permease	C
1587047	1587889	-	SMD_RS07405	MULTISPECIES: putrescine ABC transporter permease PotI	C
1587973	1589337	-	SMD_RS07410	NAD-dependent succinate-semialdehyde dehydrogenase	C
1589504	1590526	-	SMD_RS07415	magnesium and cobalt transport protein CorA	C
1590533	1591792	-	SMD_RS07420	DUF4105 domain-containing protein	C
1591789	1592670	-	SMD_RS07425	MULTISPECIES: CBS domain-containing protein	C
1592735	1593301	-	SMD_RS07430	hypothetical protein	C
1593379	1593984	-	SMD_RS07435	hypothetical protein	C

1594032	1594517	-	SMD_RS07440	MULTISPECIES: endoribonuclease YbeY	C
1594514	1595500	-	SMD_RS07445	PhoH family protein	C
1596024	1596980	-	SMD_RS07450	HlyD family efflux transporter periplasmic adaptor subunit	C
1597001	1597924	-	SMD_RS07455	ABC transporter ATP-binding protein	C
1597921	1599033	-	SMD_RS07460	ABC transporter permease	C
1599122	1600540	-	SMD_RS07465	tRNA (N6-isopentenyl adenosine(37)-C2)-methylthiotransferase MiaB	C
1600728	1601345	-	SMD_RS07470	glutathione S-transferase family protein	C
1601426	1602394	-	SMD_RS07475	WYL domain-containing transcriptional regulator	C
1602441	1603346	-	SMD_RS07480	lytic transglycosylase domain-containing protein	C
1603546	1604166	*petA*	SMD_RS07485	MULTISPECIES: ubiquinol-cytochrome c reductase iron-sulfur subunit	C
1604166	1605425	-	SMD_RS07490	cytochrome b	C
1605418	1606167	-	SMD_RS07495	MULTISPECIES: cytochrome c1	C
1606302	1606937	-	SMD_RS07500	MULTISPECIES: stringent starvation protein A	C
1607022	1607474	-	SMD_RS07505	MULTISPECIES: ClpXP protease specificity-enhancing factor	C
1607579	1607929	-	SMD_RS07510	MULTISPECIES: DUF3301 domain-containing protein	C
1607997	1608998	-	SMD_RS07515	DUF2272 domain-containing protein	C
1609179	1610030	*nadC*	SMD_RS07520	carboxylating nicotinate-nucleotide diphosphorylase	C
1610027	1610296	-	SMD_RS07525	MULTISPECIES: hypothetical protein	C
1610355	1610858	*purE*	SMD_RS07530	MULTISPECIES: 5-(carboxyamino)imidazole ribonucleotide mutase	C
1610855	1612003	-	SMD_RS07535	5-(carboxyamino)imidazole ribonucleotide synthase	C
1612116	1612457	-	SMD_RS07540	MULTISPECIES: carboxymuconolactone decarboxylase family protein	C
1612533	1613111	-	SMD_RS07545	superoxide dismutase	C
1613232	1613552	*grxD*	SMD_RS07550	MULTISPECIES: Grx4 family monothiol glutaredoxin	C
1613549	1614325	-	SMD_RS07555	SDR family NAD(P)-dependent oxidoreductase	C
1614577	1617777	-	SMD_RS07560	TonB-dependent receptor	C
1618031	1619419	-	SMD_RS07565	MULTISPECIES: LOG family protein	C

1619581	1620096	-	SMD_RS07570	prepilin-type N-terminal cleavage/methylation domain-containing protein	C
1620093	1620590	*pilV*	SMD_RS07575	MULTISPECIES: type IV pilus modification protein PilV	C
1620599	1621756	-	SMD_RS07580	prepilin-type N-terminal cleavage/methylation domain-containing protein	C
1621762	1622283	-	SMD_RS07585	pilus assembly protein	C
1622297	1626049	-	SMD_RS07590	hypothetical protein	C
1626073	1626480	-	SMD_RS07595	type IV pilin protein	C
1626657	1627202	-	SMD_RS07605	prepilin-type N-terminal cleavage/methylation domain-containing protein	C
1627331	1629355	-	SMD_RS07610	excinuclease ABC subunit UvrB	C

Table S3. MICs of several antibiotics for *S. maltophilia* tigecycline-evolved populations

	MIC (mg/L)												
Strain	**TGC**	**CAZ**	**ATM**	**SXT**	**E**	**C**	**FOS**	**CIP**	**OFX**	**NA**	**S**	**CN**	**TE**
D457	1	0.75	6	0.094	1024	8	192	4	4	4	16	1.5	2
Population A	128	0.75	96	0.125	2048	64	64	128	128	64	96	1.5	32
Population B	192	1	256	0.094	4096	128	128	128	128	96	192	2	64
Population C	96	1.5	96	0.032	4096	96	96	512	256	24	192	3	32
Population D	128	0.75	3	0.19	4096	64	64	512	256	16	64	3	48

MIC, minimum inhibitory concentration; TIG, tigecycline; CAZ, ceftazidime; ATM, aztreonam; SXT, trimethoprim/sulfamethoxazole; E, erythromycin; C, chloramphenicol; FOS, fosfomycin; CIP, ciprofloxacin; OFX, ofloxacin; NAL, nalidixic acid; S, streptomycin; CN, gentamicin; TE, tetracycline.

DISCUSSION

4. Discussion

The evolution and spread of multidrug resistant bacteria has become a major threat to modern medicine, being the infections caused by these microorganisms a significant economic and health burden [169]. To efficiently tackle this problem, it is necessary to better understand the factors and mechanisms that can potentially have an effect on the evolution, emergence and spread of antibiotic resistance.

MDR is particularly common in Gram-negative bacteria, having important clinical consequences concerning their spread and treatment options [170]. Among MDR Gram-negative bacteria, *S. maltophilia*, an important nosocomial pathogen in clinical environments, exhibits an intrinsic low susceptibility to a wide range of antimicrobial agents due to the presence of several resistance mechanisms encoded in its genome [129,135]. Besides the presence of intrinsic resistance determinants, *S. maltophilia* can also acquire resistance as a result of genetic changes that allow this bacterium to overcome the toxic effects of an antibiotic, or transiently expressing certain genes that would permit this opportunistic pathogen to resist, in a temporal and reversible way, the effect of an antimicrobial drug [171]. In all cases, MDR efflux pumps, particularly those from the RND family, are key players in the low susceptibility that *S. maltophilia* displays against a great part of the available antibiotics [172].

During the course of this PhD thesis, we have aimed our research at the study of the *S. maltophilia* inducible and acquired resistance to antibiotics, specially focusing on the role that RND efflux pumps play in both types of resistance.

4.1. *S. maltophilia* transient resistance to antibiotics

RND efflux pumps are complex biological machineries that, connecting the inner and outer membranes through the periplasm of the bacterial cell, are able to extrude a wide variety of compounds, including antibiotics [35]. The regulation of the expression of these systems is usually mediated by the action of positive and/or negative transcription factors, which are normally encoded upstream the operon coding for the efflux pump [82]. Although some RND efflux systems present low levels of basal expression, high-level expression can be achieved through the presence of an effector or a specific environmental condition that trigger the

expression of the efflux pump. This induction can be achieved directly through the binding of the specific effector molecule to the corresponding transcriptional regulator, or indirectly by the action of the inducer-derived molecules or the generated stress response [173,174]. In spite of the wide variety of compounds that are known to be substrates of RND efflux pumps, the number of effectors or cues known to trigger their expression, and potentially contribute to transient resistance, is lower in comparison [175].

Regarding *S. maltophilia*, the contribution of the RND efflux pumps to transient resistance to antibiotics has not been fully explored and only few studies regarding this topic have been carried out. For instance, it is known that the expression of *smeDEF* can be triggered by the presence of the biocides triclosan and benzalkonium chloride [69,176], as well as by some plant-derived flavonoids [68], that bind to the transcriptional repressor SmeT and subsequently change the bacterial susceptibility to quinolones. The study of the situations or signals that induce transient antibiotic resistance is valuable since bacteria can encounter these conditions during the course of an infection. However, the detection of this kind of resistance is usually complicated when classical susceptibility methodologies are used [177].

The use of fluorescence-based reporters has been widely employed for several purposes, from environmental applications, as detection of toxic pollutants [178], to monitoring gene expression, for instance, in response to the host environment [179]. In this thesis, we have developed YFP-based reporters of the expression of two RND efflux pumps, SmeVWX and SmeYZ, with the purpose of finding conditions and/or molecules that trigger the expression of these *S. maltophilia* efflux systems. To this end, two screenings of potential effector compounds of the expression of either *smeVWX* or *smeYZ* were performed.

4.1.1. RND efflux pumps as stress-induced determinants

The first screening was carried out by measuring the fluorescence levels produced by the *smeVWX* reporter strain during the exposure to 29 compounds from different categories (antibiotics, biocides, heavy metals, etc.) at several concentrations. A *S. maltophilia* strain harbouring the *smeVWX* reporter was grown in the presence of the selected compounds in a microtiter plate, and fluorescence and growth were recorded over time. This methodology allowed us to identify vitamin K_3 as a potential inducer of the *smeVWX* expression, which was confirmed by real-time PCR. Moreover, two structural analogues of vitamin K_3, vitamin K_2 and

plumbagin, which belong to the naphthoquinones family as well [180], were also found to increase *smeVWX* expression in a concentration-dependent way.

In the second screening, we improved the previous approach in order to analyse a much wider range of compounds: the Biolog phenotype microarrays. These microarrays are 96-well microtiter plates, each one containing 24 toxic compounds at 4 different concentrations. The Biolog plates have been developed for performing a metabolic fingerprinting of bacterial strains, testing the effect of genetic changes, particularly knock-outs, under nearly 2,000 chemical and nutrient conditions [181]. In our case, we combined this high-throughput technology together with the *smeYZ* sensor, as well as with the previously tested sensor corresponding to *smeVWX*, to analyse 144 different compounds as potential inducers of these systems. A *S. maltophilia* strain harbouring a reporter plasmid that constitutively produces YFP was used as a reference to decipher the induction situations of *smeVWX* and *smeYZ*. Among all the tested compounds, iodoacetate, clioquinol and sodium selenite were identified as *smeVWX* inducers, while in the case of *smeYZ*, boric acid and the antibiotics erythromycin, chloramphenicol, and lincomycin, were recognized as potential inducers. Induction was confirmed by performing flow cytometry and real-time PCR.

RND-type efflux systems have been mainly studied in Gram-negative bacteria as antibiotic resistance determinants [182,183]. However, they are involved in other physiological processes that are important for the bacterial cell, such as detoxification of secondary metabolites or toxic molecules, survival in the host environment, biofilm formation, or cell-to-cell communication [184]. In addition, a substantial number of efflux pumps are stress-inducible, being able to respond to a plethora of stress conditions (ribosome disruption, the presence of reactive oxygen species, membrane-damaging agents, nutrient limitation, etc.). This fact indicates that at least some of these efflux systems have evolved to respond to environmental stresses, thus working as components of the organism's stress response [185]. For instance, the expression of a number of efflux pumps and/or their regulatory elements, is modulated by oxidative stress agents. Thus, these efflux systems could be involved in the amelioration of the detrimental conditions generated by these products [186-188]. We propose that if RND efflux pumps participate as stress determinants in *S. maltophilia*, a common inducer mechanism could be identified by analysing the effector compounds that trigger their expression.

About SmeVWX, the identification of vitamin K_3 and its analogues as inducers, which are known to generate oxidative stress, led us to the idea that oxidative stress might be the signal triggering the expression of *smeVWX*. However, the generator of oxidative stress tert-butyl hydroperoxide did not lead to an increment of the efflux pump expression, hence suggesting that just oxidative stress could not be the inducer signal of *smeVWX*. The results from the second screening, which included many more compounds, shed some light on the possible *smeVWX* inducer cue, since the new identified compounds, iodoacetate, clioquinol and sodium selenite, as well as vitamin K_3, are thiol-reactive compounds, that is, they are able to interact with thiol groups on proteins causing different effects [180,189-191]. Although the molecular mechanism by which *smeVWX* expression is triggered is not fully understood, our data suggest that the induction of this efflux pump is associated with the thiol reactivity of the identified molecules.

Regarding SmeYZ, boric acid and a set of antibiotics including erythromycin, chloramphenicol, and lincomycin, were identified as inducers. The three inducer antibiotics are known to inhibit the protein synthesis through different mechanisms involving the 50S subunit of the ribosome [192-194]. Although boric acid is not an antibiotic, is has been reported that it impairs the tRNAs acylation, inhibiting protein synthesis [195]. Other compounds identified from the Biolog microarrays that led to an overexpression of *smeYZ* (e.g. fusidic acid, tylosin, spiramycin, oleandomycin, etc.), are also known inhibitors of the protein synthesis. All the reported findings suggest that the mechanism underlying *smeYZ* induction is associated with protein synthesis inhibition. Indeed, it has been previously reported that ribosome-targeting antibiotics are able to trigger the expression of the *P. aeruginosa* MexXY efflux pump [196]. In *S. maltophilia*, it is possible that SmeYZ plays a role in exporting anomalous peptides or processed by-products, thus alleviating the ribosome-stalling stress. However, the molecular basis behind this mechanism is still unknown.

4.1.2. Inducers are not necessarily substrates of RND efflux pumps

A common feature of some RND efflux pumps is the ability to extrude some of the inducer molecules that trigger their expression as a mechanism of protection against the toxicity of such molecules. For instance, bile salts are both inducers and substrates of the AcrAB efflux pump of *Salmonella* [197]; and triclosan induces and is extruded by the *S. maltophilia* SmeDEF efflux pump [69]. Concerning our recently identified compounds, we showed that only vitamin K_3 acts as a possible substrate of SmeVWX, since bacterial growth in the presence of this

inducer is impaired in the absence of the efflux protein SmeW. Vitamin K_3, a compound that has been isolated from phanerogams and fungi [198], has been reported to have a role in the defence against plant-pathogens [199,200]. Since *S. maltophilia* is able to colonize plant roots, SmeVWX efflux pump could be involved in the detoxification of noxious plant-derived compounds, as menadione, which would be produced by plants during pathogenesis and would act as a stress inducer factor.

None of the other inducers of *smeVWX* or *smeYZ* seems to be substrate of their respective efflux pumps, due to the fact that the strains lacking either SmeW or SmeZ do not show differences in growth during exposure to the toxic inducer molecules. As a consequence, here we propose that not all the RND efflux pump inducers are necessarily their substrates as well. This can be explained through the fact that bacteria might employ other detoxifying determinants in order to counteract the toxicity generated by the inducers. We also hypothesize that inducer compounds might not always be efflux pump substrates due to the cell compartmentalization. That is, the induction processes occur in the cytoplasm, while RND efflux pumps principally extrude the molecules from the inner membrane or from the periplasm of the cell [201]. Thus, if the inducer molecule is degraded or extruded by other determinants, or if it remains in the cytoplasm, it can justify why these inducers are not being extruded by the efflux systems whose expression they trigger.

4.1.3. Inducer-triggered transient resistance to antibiotics

The study of RND efflux pumps inducers in *S. maltophilia* is also relevant from a clinical point of view, since overexpression of these resistance determinants during exposure to their respective inducers could lead to a transient situation of antibiotic resistance. Our results show that sodium selenite is able to promote transient resistance against the antibiotic ofloxacin, a substrate of SmeVWX; and vitamin K_3, besides leading to transient resistance towards ofloxacin, it also does against chloramphenicol. Besides, transient resistance is also achieved for amikacin (a SmeYZ substrate) when *S. maltophilia* is exposed to the *smeYZ* inducer lincomycin, being an example of an antibiotic triggering resistance against a different antibiotic. In the case that bacteria are exposed to these external compounds in a treated patient, it could potentially increase the resistance levels, thus challenging therapy. For instance, vitamin K_3 and sodium selenite, due to their cytotoxic effects, show good antitumor activity against various human cancer cells [202,203]. Because of this, vitamin K_3, as well as some of its analogues, have

been used alone or in combination with other chemotherapeutic drugs in several *in vitro* and *in vivo* studies for the treatment of different types of cancer [204-208]. Likewise, sodium selenite has also been administered to terminal cancer patients during a phase I clinical trial [209]. Since *S. maltophilia* usually causes infections in immunocompromised patients, as those affected with cancer, the presence of these compounds during an infection could lead to efflux pump overexpression and transient antibiotic resistance, which may complicate the treatment course.

4.2. *S. maltophilia* acquired resistance to antibiotics

During the last decades, we have witnessed one of the most fast and dramatic events of biological evolution of anthropogenic origin: the adaptation of bacteria to antibiotics. This adaptation can be the result of acquisition of new exogenous genetic material, as well as mutations or genome rearrangements that occur in the bacterial chromosome and modify the pre-existing genes [210]. Understanding the dynamics through which bacteria develop resistance to antibiotics, as well as knowing the parameters that determine the persistence and spread of acquired resistance, is essential for making an effective use of antibiotics.

A methodology that enables the study of adaptation under controlled laboratory settings is the performance of experimental evolution assays [211]. The longest bacterial evolution experiment was initiated by Lenski in 1988 [212], in which more than 60,000 generations were reached in the last analysis [213]. In this study, which is currently in progress, the genetic dynamics and fitness of several daily-passaged *E. coli* populations are determined to study long-term adaptation to a constant environment [213]. Regarding antibiotic resistance, exposure to antimicrobial drugs implies an environmental change that influences the evolutionary dynamics of bacterial populations [54,214]. In this respect, experimental evolution in the presence of antibiotics is used as a practical application for predicting the evolution towards resistance to antibiotics that are currently used at clinics, as well as to new antimicrobial drugs that are under development [54,215-217].

S. maltophilia is known to acquire multidrug resistance rapidly after exposure to different antimicrobials [218]. However, most of the studies concerning acquisition of resistance in this bacterium are performed using resistant clinical isolates or through the isolation of colonies from plates with high antibiotic concentration [152,219]. Consequently, little information is available about the genetic dynamics and phenotypic effects derived from the acquisition of

antibiotic resistance in *S. maltophilia* populations challenged with variable antibiotic concentrations overtime. During the course of this thesis, we have performed experimental evolution experiments exposing *S. maltophilia* to increasing concentrations of the antibiotics ceftazidime or tigecycline, both appropriate alternatives for the treatment of *S. maltophilia* infections, with the aim of revealing new potential genetic mechanisms underlying the acquisition of resistance to these antibiotics, as well as the evolutionary trajectories involved. The results derived from both experimental evolutions have led to the discovery of different mechanisms potentially involved in the resistance phenotype observed in this opportunistic pathogen after such evolutions.

4.2.1. Adaptation to ceftazidime and the role of SmeGH efflux pump in resistance and bacterial physiology

In the case of ceftazidime evolution, acquisition of resistance against this beta-lactam by *S. maltophilia* seems to be mainly associated with the extrusion of the antibiotic through efflux systems. Particularly, the four evolved populations present two different mutations in the gene coding for the transporter protein of the SmeGH efflux pump, *smeH*, and two of them have mutations in genes encoding the auxiliary (*yrbC*) and the permease components (*yrbE*), respectively, of an uncharacterized ABC transporter. In addition to these efflux-associated genes, three of the populations show different mutations in the gene coding for the two-component sensor histidine kinase *phoQ*, whose role in beta-lactams resistance has not been studied deeply, but is known to be involved in polymyxin and antimicrobial peptides resistance in other bacterial species [220,221]. Ceftazidime exposure also led to the acquisition of genetic changes in the genes encoding four hypothetical proteins, as well as in the peptidoglycan transpeptidase-coding gene, *ftsI*, which has been previously associated with resistance against beta-lactam drugs [52,222].

Among all the identified *S. maltophilia* RND efflux pumps, some of them have been well characterized and their role in resistance and/or virulence have been described [144,146-149,155]. Regarding SmeGH, a recent work has reported that the overexpression of this efflux pump contributes to the acquisition of resistance towards fluoroquinolones. However, the mutation(s) leading to the observed phenotype are not elucidated [151]. Concerning our study, the fact that both mutations have been selected in the efflux transporter protein, but not in a regulatory element, which is the most common event when associating acquired resistance and RND efflux

pumps, makes the study of this efflux system very interesting. Hence, we constructed strains carrying both ceftazidime-selected amino acid substitutions (P326Q and Q663R), independently and in combination, to study their particular contribution to ceftazidime resistance. Our results show that the presence of both mutations in *smeH* confers higher levels of resistance to ceftazidime in comparison with the unique presence of P326Q. Surprisingly, when present alone, the second amino acid substitution Q663R does not confer any change in ceftazidime susceptibility, that is, this mutation is neutral in the wild-type background, regarding ceftazidime susceptibility. This result, together with the fact that Q663R only emerges in the evolution when P326Q is already present, can be interpreted as a possible epistasis event, in which the effect of Q663R on ceftazidime resistance is dependent on the genetic background. This kind of epistasis (sign epistasis) can limit the evolutionary paths available to a population, having profound effects on the evolution trajectory [96].

S. maltophilia RND efflux pumps can be involved in the three types of resistance that have been previously described. Our results performed with a *smeH*-deficient mutant show that this efflux pump seems to be an important intrinsic resistance determinant for several antibiotics, including not only beta-lactams, but also quinolones, polymyxin B and tetracycline. Besides antibiotics, SmeGH efflux pump could be a detoxification element for other toxic compounds, due to the hypersusceptibility phenotype that this mutant displays against the previously described vitamin K_3, the oxidative stress agent tert-butyl hydroperoxide, the biocide benzalkonium chloride, and the plant-derived compound naringenin. Besides, the *smeH*-deficient strain shows an enhanced capacity of biofilm formation, suggesting that SmeGH negatively contributes to the formation of biofilm in *S. maltophilia*. It is possible that this RND efflux pump regulates somehow biofilm-related genes, as in the case of the *Listeria monocytogenes* ABC transporter lm.G_1771, which negatively regulates the expression of cell surface proteins involved in biofilm formation [223]. Another explanation is that SmeH deletion could lead to the accumulation of toxic compounds, or metabolic by-products, that could trigger a stress response and the enhancement of the expression of the biofilm-related genes, as happens in *Burkholderia cenocepacia* [224]. Overall, these data indicate that *S. maltophilia* SmeGH efflux pump plays a wider role than just in antibiotic resistance, influencing additional features, such as biofilm formation, that are fundamental for bacterial pathogenesis.

4.2.2. Adaptation to tigecycline: from SmeDEF efflux pump to several genetic determinants

Concerning tigecycline evolution, acquisition of resistance seems to be a more complex process since it involves the participation of several genes: mutations in antibiotic extrusion-associated genes, ribosome-related genes, genes involved in the LPS biosynthesis and membrane homeostasis, and even large chromosomal rearrangements. In this case, the efflux pump-associated mutation selected in the four evolved populations is present in *smeT*, which encodes the negative transcriptional regulator of the SmeDEF efflux pump. This result suggests that *smeDEF* overexpression leads to the increased extrusion of tigecycline outside the bacterial cell, rendering bacteria less susceptible to this antibiotic. It is important to highlight the fact that both amino acid substitutions selected in SmeT after tigecycline exposure (L166Q and T197P) have been previously associated with antibiotic resistance due to *smeDEF* overexpression in clinical isolates, supporting the notion that experimental evolution results can be extrapolated to the clinical settings [225-228].

Several mutations were also detected in some ribosome-related genes, such as the ribosome recycling factor (*frr*), and the 30S ribosomal proteins S10 (*rpsJ*), S21 (*rpsU*) and S1 (*rpsA*). Since tigecycline, as other tetracyclines, binds to the bacterial 30S ribosome subunit, blocking the entry of transfer RNA and preventing protein synthesis, mutations in the target-related genes are expected as a protective mechanism [229]. Mutations in *frr* have been previously found in a similar experimental evolution performed in *P. aeruginosa* in the presence of tigecycline [51]; and *rpsJ* has been designated as a relevant marker for detecting tigecycline resistance due to the fact that mutations in this gene have been identified in other pathogenic bacteria after tigecycline experimental evolutions, as well as in non-susceptible tigecycline clinical isolates [230-232]. Furthermore, a mutation in *rpsJ* has been recently found in a tigecycline-resistant *Klebsiella pneumoniae* clinical isolate from a patient undergoing tigecycline therapy, pointing that *rpsJ* mutations can be selected *in vivo* during tigecycline exposure [233]. Regarding *rpsU* and *rpsA*, this is the first report where these genes are potentially related to tigecycline resistance.

A third set of genes related to the LPS biosynthesis and membrane homeostasis were found mutated after tigecycline challenge. These genes code for the lipid A biosynthesis enzymes lauroyl acyltransferase HtrB and the phosphoethanolamine transferase SMD_RS16325, the UDP-glucose dehydrogenase Ugd, the two-component sensor histidine

quinase PhoQ, and the diacylglycerol kinase DgkA. Except for *ugd* and *dgkA*, whose role in resistance has not been reported yet, the other LPS-related genes have been previously identified as contributors to antibiotic and/or antimicrobial peptides resistance in other bacterial pathogens, as in *P. aeruginosa* [220,234] and *N. gonorrhoeae* [235]. Mutations in genes from the LPS biosynthesis pathway, but different than the ones found in our study, have also been identified in spontaneous *E. coli* mutants with low susceptibility to tigecycline [236]. Although the relationship between LPS modification and acquisition of tigecycline resistance is unclear, we propose that the observed phenotype could be due to an impairment of tigecycline to diffuse through the membrane inside the cell (assuming that tigecycline follows the same entrance mechanism as tetracycline [236,237]) as a result of the outer membrane modification. Mutations in enzymes related to other important bacterial processes, and previously described to have a role in resistance and/or virulence in other species were also detected, as in the S-adenosylmethionine decarboxylase (SpeD) [238], or in the *dsbB* gene, an element of the DsbA/DsbB system [239]. Nevertheless, the mechanism through which they contribute to tigecycline resistance needs further study.

Besides SNPs and small insertions/deletions, two inverted duplications of 300- and 60-kbp were detected in two of the tigecycline-evolved populations, respectively. Both regions, which share 63 common genes, contain a great variety of genetic determinants with diverse functions, as transcriptional regulators, transporters or enzymes. Since both inverted-and-duplicated sequences are located within the same region of the *S. maltophilia* chromosome and share 63 genes, we suggest that these chromosomal rearrangements provide an advantage to the bacterium, either by contributing to tigecycline resistance, or by providing a fitness cost compensation.

4.2.3. Evolutionary trajectories and the importance of RND efflux pumps towards ceftazidime and tigecycline resistance

Reconstruction of the evolutionary dynamics of all the evolved lineages allowed us to highlight the striking similarities they share: the first step towards ceftazidime resistance in the four evolved populations is the P326Q amino acid substitution in SmeH, while the first genetic change identified in the tigecycline-evolved populations are two different amino acid substitutions in the SmeDEF regulator SmeT. For all the ceftazidime-evolved populations, the following mutations towards resistance occur from the 20th day of evolution; while for those

populations undergoing tigecycline exposure, mutations emerged along the whole evolution period, being *frr* the next in appearing mutated for most cases after *smeT*.

These results highlight the importance that RND efflux pumps have in the adaptation towards antibiotics in *S. maltophilia*. This RND efflux pump-mediated acquired resistance is achieved through different mechanisms depending on the location of the mutations. Concerning the SmeGH efflux pump, the *in silico* analysis of its structure allowed us to localize the P326 residue in the vicinity of the deep binding pocket of the SmeH-homolog AcrB from *E. coli*, which was used as a model for mapping the *S. maltophilia* SmeH residues. In addition, the *in silico* mutagenesis performed on this protein structure for predicting the impact that the P326Q change could have in the binding pocket conformation, shows that the Q326 residue is pointing towards the base of the binding cavity. Although the change in the residue orientation does not seem to directly affect the interaction between the SmeH binding pocket and the antibiotic substrate, we propose that it could interact with other residues that constitute the binding pocket and affect the recognition of the substrates, thus ameliorating their extrusion. Furthermore, the second mutated residue in SmeH, Q663, which corresponds to the V672 in AcrB, is located at the bottom of the predicted access pocket, which would be involved in the initial stage of drug binding [240]. All these data suggest that ceftazidime resistance is in part achieved as a result of a change in the recognition and/or accommodation of the substrate, in this case ceftazidime, inside the transporter protein. This fact would also explain the reason why the Q663R substitution only confers ceftazidime resistance when P326Q is present. We suggest that modifications just in the access cavity, where Q663R is predicted to be located, could not be sufficient to cause an effect in the extrusion of the substrates. Nevertheless, when P326Q is present, and antibiotic binding supposedly ameliorated, both access and recognition of the antibiotic would be enhanced, resulting in an improved substrate extrusion. Conversely, in the case of SmeDEF, as mutations arose in the negative transcriptional regulator-coding gene *smeT*, we propose that the first step towards tigecycline resistance acquisition in *S. maltophilia* is the result of *smeDEF* overexpression, leading to an increased efflux of tigecycline outside the cell. Hyperproduction of RND efflux pumps, including SmeDEF, as a consequence of mutations in the regulatory elements, has been previously reported to contribute to the acquisition of resistance in *S. maltophilia* [47,146,150,154,225,226]. However, the presence of amino acid substitutions in SmeH, allows us to report, for the first time, on the selection of mutations occurring in an efflux pump transporter protein after antibiotic exposure in *S. maltophilia*.

Although high-level resistance to both antibiotics has been acquired through the accumulation of mutations in different genetic elements, the fact that some of the evolved lineages share common aspects indicates that evolution towards either ceftazidime or tigecycline resistance is, to a certain degree, a deterministic process driven by the selective pressure and suggests that evolution can be more predictable than expected.

4.2.4. Cross-resistance and collateral sensitivity as a consequence of acquisition of ceftazidime or tigecycline resistance

Acquisition of resistance against one antibiotic can imply a variation in the susceptibility towards other antibiotics (cross-resistance or collateral sensitivity), from the same and/or different family. This possibility was examined for the reconstructed mutants carrying the SmeH substitutions, either alone or in combination, showing that, besides contributing to ceftazidime resistance, the presence of P326Q and the combination of P326Q/Q663R, also decrease the susceptibility to other antibiotics, particularly beta-lactams. However, the presence of just Q663R, does not change neither the susceptibility to ceftazidime nor to other beta-lactams, but it does for tetracycline, suggesting that such mutation could be selected under tetracycline exposure without the presence of P326Q. The location of both P326Q and Q663R amino acid substitutions in the proximities of the predicted binding and access pockets of the efflux pump transporter, respectively, could lead to cross-resistance not only to ceftazidime, but also to other antimicrobials that are substrates of the efflux pump due to an amelioration of their extrusion. This picture is supported by the fact that the susceptibility to the other tested toxic compounds also changes in these strains.

Regarding tigecycline evolution, cross-resistance and collateral susceptibility to other antibiotics was assessed in the four last-step tigecycline-evolved populations. The fact that *smeDEF* is overexpressed due to *smeT* mutations can explain the observed cross-resistance displayed by all the populations against quinolones, chloramphenicol, erythromycin and tetracycline, since they are SmeDEF substrates [147]. Mutations in some ribosome-related genes, which are also tetracycline targets, may also explain the observed cross-resistance to this antibiotic. Besides these similarities, the different mutational trajectories followed by each independent line have also led to some differences, such as the higher susceptibility observed for trimethoprim/sulfamethoxazole in two of the populations. It is also important to highlight the observed collateral sensitivity to fosfomycin for all the tigecycline-evolved populations.

Although the mechanism by which this phenotype occurs remains unclear, it is relevant to consider a combination of fosfomycin as an antimicrobial agent for the treatment of tigecycline-resistant *S. maltophilia* strains. Conversely, combination treatments including tigecycline and one of the antibiotics to which cross-resistance is observed, as quinolones, should be disregarded.

4.2.5. Fitness costs associated to the acquisition of ceftazidime or tigecycline resistance

The fate of a resistance mutation in a bacterial population is in part determined by the changes in the fitness associated to its presence. While mutations that impose little or no fitness cost are more probably to persist in the population in the absence of antibiotic, those resistance mechanisms that impose a fitness burden, typically observed as a reduction of bacterial growth rate, drive resistant bacteria to be outcompeted by their susceptible counterpart in an antibiotic-free medium [87,92]. Thus, fitness cost is a key parameter that influences the evolution and spread of antibiotic resistance [86].

The fitness analysis for the *S. maltophilia* SmeH mutants, measured as growth in the absence of antibiotic, shows that the presence of *smeH* mutations, alone or in combination, does not lead to a cost in fitness. However, the absence of the efflux pump transporter implies a more deficient growth, which suggests that this intrinsic resistance determinant may be under positive selection pressure. The fact that the second amino acid substitution in SmeH emerged days after the first one, and did not cause ceftazidime resistance by itself, led us to think that this mutation could be a compensatory mutation selected to counteract a potential cost of the first one. However, this idea is discarded since the presence of P326Q does not suppose an impairment in growth, at least in the laboratory tested conditions. It has been reported that no-cost (or low-cost) mutations are usually selected in clinical isolates [241-243]. This fact makes that, in the absence of antibiotic treatment, bacteria harbouring these no-cost mutations are more likely to be maintained in the population.

Regarding acquisition of tigecycline resistance, fitness was measured at different time points in the four evolved populations. In this case, as mutations accumulate and susceptibility against tigecycline decreases, bacterial fitness gets more compromised in the absence of antibiotic in comparison with the wild-type strain during the evolution process. Although less drastic than in the populations, the individual clones isolated from each evolved lineage at

different time points also exhibit a fitness burden along time. Although the majority of the selected mutations might have a potential role in tigecycline resistance, we postulate that some of these genetic changes, including both inverted duplications, may have been selected in order to compensate the costs derived from other mutations, since, in some cases, the fitness cost seems to be recovered between two continuous time points in both the populations and the single clones. These results point that, in the absence of selective pressure, tigecycline-resistant strains containing these mutations in their genome are less probable to be maintained in the whole population.

The fact that SmeH substitutions drive to no fitness cost while the tigecycline-evolved populations and clones present an impairment in growth, demonstrates that the cost of resistance can be highly variable and highlights that fitness effects are strongly dependent on the genetic background [244]. While in the first case fitness is measured in the context of the recreated strains, in the second situation, growth rate is determined in the whole population setting, as well as in single clones isolated from that populations, which may have different non-detected mutations that can influence the measurement of this parameter. Thus, the cross-talk between the resistance mutation and the genetic background, including other resistance or compensatory mutations, can remarkably modulate the resistance-derived costs.

4.2.6. Concluding remarks

The results derived from this thesis provide important information concerning the prediction of the acquisition of antibiotic resistance by *S. maltophilia*, both through the transient expression of RND efflux pumps and via the selection of antibiotic resistance mutations. We have found numerous inducer compounds of the expression of two of these efflux systems by the use of fluorescent-based strains. This methodology can be applied to monitoring the expression of other types of efflux pumps of this bacterium and other bacterial species, being an interesting topic for future work. In addition, we have elucidated the main *S. maltophilia* mechanisms of tigecycline and ceftazidime resistance acquisition by experimental evolution studies, showing that RND efflux pumps play a decisive role. The clinical implications of the appearance of these resistance mutations under antibiotic treatment *in vivo* is a question for future research. However, the presence of some of these mutations in bacterial clinical isolates highlights the relevance of experimental evolution studies to understand the potential mechanisms involved in the acquisition of antibiotic resistance by bacterial pathogens.

CONCLUSIONS

5. Conclusions

I. The use of the YFP-based *S. maltophilia* reporter strains allows the quantification and analysis of the expression driven by the promoters of SmeVWX and SmeYZ RND efflux pumps.

II. The Biolog phenotype microarray technology can be used as a high-throughput tool for the identification of MDR efflux pump inducers.

III. Vitamin K_3, and its analogues vitamin K_2 and plumbagin, as well as iodoacetate, clioquinol and sodium selenite are *smeVWX* inducers, suggesting that thiol reactivity is the signal that triggers *smeVWX* expression.

IV. Boric acid, erythromycin, chloramphenicol and lincomycin are *smeYZ* inducer compounds, pointing that protein synthesis inhibition is the signal underlying the induction of *smeYZ.*

V. Excepting vitamin K_3, which seems to be a substrate of SmeVWX, the remaining identified inducer compounds of both SmeVWX and SmeYZ do not seem to be extruded by the efflux pump they induce, indicating that not all MDR efflux pumps inducers are substrates of these systems.

VI. The overexpression of *smeVWX* and *smeYZ* triggered by their inducer molecules leads to transient resistance to antibiotics that are known substrates of one or another efflux system.

VII. The first step leading to the acquisition of resistance to ceftazidime is achieved by two amino acid substitutions in the SmeGH efflux pump transporter protein, SmeH, possibly due to modifications in the antibiotic recognition and binding cavities.

VIII. The genetic background is of relevance regarding the contribution of each SmeH amino acid substitutions to ceftazidime resistance, since Q663R only changes ceftazidime susceptibility when P326Q is already present.

IX. SmeGH RND efflux pump is a relevant intrinsic resistance determinant of *S. maltophilia* for several antibiotics, including beta-lactams, quinolones, polymyxin B and tetracycline, as well as for other toxic compounds. SmeGH could also negatively regulate biofilm formation.

X. The first step towards acquisition of tigecycline resistance is *smeDEF* overexpression due to mutations in the negative regulator SmeT. Ribosome-, LPS biosynthesis- and membrane homeostasis-related genes are also involved in the tigecycline acquired resistance.

XI. Experimental evolution in the presence of either ceftazidime or tigecycline implies variation in the susceptibilities to other antibiotics, from the same and different families, indicating that the selected resistance mechanisms are not specific of the antibiotic which the evolution has been performed with.

XII. Whereas ceftazidime-resistance SmeH amino acid substitutions do not lead to a fitness burden in *S. maltophilia*, the populations challenged with tigecycline present a more compromised fitness in the absence of antibiotic.

XIII. The similarities present in the evolutionary trajectories of all the lineages in each of both experimental evolutions highlight the importance of RND efflux pumps in the acquisition of antibiotic resistance in *S. maltophilia* and suggest that mutation-driven evolution towards ceftazidime and tigecycline resistance evolution can be largely predictable.

6. Conclusiones

I. El uso de cepas reporteras de *S. maltophilia* basadas en la proteína fluorescente amarilla (YFP) permite la cuantificación y el análisis de la expresión debida a los promotores de las bombas de expulsión RND SmeVWX y SmeYZ.

II. La tecnología *Biolog phenotype microarray* puede usarse como una herramienta de alto rendimiento para la identificación de compuestos inductores de las bombas de expulsión múltiple de drogas (MDR).

III. La vitamina K_3 y sus análogos, la vitamina K_2 y la plumbagina, así como el yodoacetato, el clioquinol y el selenito de sodio, son compuestos inductores de *smeVWX*, lo que sugiere que la reactividad del tiol es la señal que desencadena la expresión de *smeVWX*.

IV. El ácido bórico, la eritromicina, el cloranfenicol y la lincomicina son compuestos inductores de *smeYZ*, lo que indica que la inhibición de la síntesis de proteínas es la señal subyacente a la inducción de *smeYZ*.

V. A excepción de la vitamina K_3, que parece ser un substrato de la bomba SmeVWX, los restantes compuestos inductores identificados, tanto para la bomba SmeVWX como para SmeYZ, no parecen ser expulsados por la bomba que inducen, indicando que no todos los inductores de bombas MDR son substratos de estos sistemas.

VI. La sobre-expresión de *smeVWX* y de *smeYZ* originada por sus compuestos inductores genera una resistencia transitoria a los antibióticos que son substratos de uno u otro sistema de bombeo.

VII. El primer paso hacia la adquisición de resistencia a ceftazidima implica dos substituciones de aminoácido en la proteína transportadora de la bomba de expulsión SmeGH, SmeH, posiblemente debido a modificaciones en las cavidades de reconocimiento y de unión del antibiótico.

VIII. El fondo genético tiene relevancia en lo que respecta a la contribución de cada una de las substituciones de aminoácido de SmeH a la resistencia a ceftazidima, puesto que Q663R sólo modifica la susceptibilidad a este antibiótico cuando P326Q está ya presente.

IX. La bomba de expulsión SmeGH, de la familia RND es un determinante de relevancia en la resistencia intrínseca de *S. maltophilia* a varios antibióticos, incluyendo beta-lactámicos, quinolonas, polimixina B y tetraciclina, así como para otros compuestos tóxicos. SmeGH también podría regular negativamente la formación de biopelículas.

X. El primer paso hacia la adquisición de resistencia a tigeciclina es la sobre-expresión de *smeDEF*, debido a mutaciones en el regulador negativo *smeT*. Algunos genes relacionados con el ribosoma, así como con el lipopolisacárido y con la homeostasis de la membrana, también están involucrados en la adquisición de resistencia a tigeciclina.

XI. La evolución experimental en presencia de ceftazidima o de tigeciclina implica una variación en la sensibilidad a otros antibióticos, de la misma y de diferente familia, indicando que los mecanismos de resistencia seleccionados no son específicos del antibiótico con el cuál se ha realizado la evolución.

XII. Mientras que las substituciones de aminoácido de SmeH que dan lugar a resistencia a ceftazidima no conllevan un coste de *fitness* en *S. maltophilia*, las poblaciones que han evolucionado en presencia de tigeciclina presentan un *fitness* más comprometido en ausencia de antibiótico.

XIII. Las similitudes presentes en las trayectorias evolutivas de cada una de las poblaciones evolucionadas destacan la importancia de las bombas RND en la adquisición de resistencia a los antibióticos en *S. maltophilia* y sugieren que la evolución de resistencia a ceftazidima y tigeciclina mediante mutaciones puede ser en gran medida predecible.

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